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13. ABSTRACT ***(Maximum 200 Words)***

Besides using immunoafffinity chromatography techniques to show that the deregulation of the cell cycle machinery in breast cancer cells, we have been studying the mechanism of genomic instability in breast cancer cells in a multifaceted manner. A few novel interactions were detected. In this study we found that p21 not only interact with PCNA but also interact directly with DNA polymerase δ p50 subunit. The tumor suppressor Rb also interacts with pol δ. Using yeast two hybrid and proteomic approaches we also found a few novel proteins that interact with polymerase δ and Replication Factor C subunits using MCF10 A and MCF 7 breast cancer cell lines. These novel findings broaden our traditional thinking about the regulation of DNA replication, repair and recombination. These studies provide a deeper understanding of the linkage between the regulation of pol δ and its accessory proteins and carcinogenesis in breast cancer.

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(4) Introduction

The major hypothesis that underlies the proposed studies is that defects in DNA polymerase δ and its accessory proteins could contribute to the molecular etiology of sporadic and hereditary breast tumors.

We have recently shown that human pol δ consists of at least four subunits (Mo et. al., 2000, Liu et al., 2000, Liu et al., submitted). We have isolated genomic DNA clones covering the gene for human pol δ p125 catalytic (POLD1) and p50 (POLD2) subunits (Chang et al., 1995, Perez et al., 2000).

We have been using a pol δ immunoaffinity column and immunoprecipitation studies to link DNA replication to the cell cycle. Understanding the cell cycle leads to greater understanding of cancer. Tracking down new cell cycle genes and studying their function in the cell cycle will help to increase our understanding of this basic biological process at both the molecular and genetic levels. The goals of these studies were to investigate the involvement of pol δ in the cancer of the breast. Potential changes in the pol δ system could involve mutations that affect its proofreading functions, leading to a mutator phenotype, as well as alterations that affect its cell cycle regulation or its ability to perform DNA repair.

5. Body

During the funded grant period we have accomplished the following:

Technical Objective 1. Determining the activity, protein and mRNA levels of pol δ PCNA, RPA, RFC in normal and breast cancer cell lines and tissues before and after challenge with DNA damaging agents.

Task 1. Assay of DNA polymerase and exonuclease activities , protein and mRNA levels.

We measured the message level of the p125 catalytic subunit of pol δ in MCF7 breast cancer cells after treatment with 30 μM N'-methyl N'nitro-N-nitrosoguanidine (MNNG) and 100 μg/ml methyl methanesulfonate (MMS). Different time points were taken at 0, 0.5, 1, 2, 3, 4 and 6 hours. We observed that the message level of p125 declined after treatment with MMS. In the same experiments we also determined the protein levels of p53, which increased after damage treatment as expected. The levels of pol δ protein were determined by Western blot in MCF7 cells treated with MMS and MNNG. The pol δ protein decreased from 0 , 0.5, 1, 2, 3, 4 and 6 hours as the p53

protein level increases after treatment with 100 μg/ml MMS. The same is true after treatment with MNNG .

We have cultured MCF10A cells to about 80% confluency. The cells were then serum starved for two hours The cells were treated with MMS or MNNG at different concentrations for four hours. The cells were harvested, lysed and the protein concentrations were determined. 20 ug of total protein were run on SDS-PAGE and Western blotted with antibodies to p125, PCNA, p53 and actin. The results showed that p53 protein increases in response to MMS treatment. p125 protein level decreased when cells were treated with MMS, which is consistent with the previous data that p53 inhibits the transcription of the polymerase δ POLD1 gene. Our results showed that both message and proteins levels of the pol δ catalytic subunit are down regulated in MCF10A and MCF7 cells while the p53 protein level increases upon treatment with MMS.

The significance of these findings is that we have shown that a cellular response to DNA damage includes a transcriptional repression of the POLD1 gene. Evidence for a mechanism for this decrease is described in studies below. There may be a link between the increase of p53 and the decrease in polymerase δ message. More direct evidence for this linkage is presented in the studies below which show that the POLD1 gene is a transcriptional target for repression by p53.

Task 2. Comparison of DNA replication functions of purified pol δ and PCNA of normal and breast cancer cells.

Experiments in this laboratory have shown that the expression of pol δ p125 mRNA increased three fold at the G1/S border in Molt 4 cells. Polymerase δ is a phosphoprotein that is most actively phosphorylated during the S phase. (Zeng et al., 1994). The amino acid sequence of the p125 catalytic subunit of DNA polymerase δ contains sites that are potential targets for different cdk kinases. These include six sites possessing the (S/T)P motif for the cdks (Ser 207, Ser788 and Thr 83, 150, 238 and 64).

Using commercially available antibodies to Cdk2 and Cdk4 we have found that p125 co-immunoprecipitates with these cell cycle dependent kinases in both MCF10A and MCF7 cells .

We had developed an immunoaffinity column to purify DNA polymerase δ (Mo et al,2000, Liu et al., 2000). We used this column to isolate pol δ holoenzyme and multiprotein complexes that interact with pol δ. We have succeeded during this grant period to scale down the preparation so that we can compare the DNA replication functions of normal and breast cancer cells with the least amount of material possible.

Preliminary data showed that we can recover DNA polymerase δ activity from both MCF10A and MCF7 cell extracts. Western blot with various antibodies was performed on proteins from fractions across the peak of polymerase activity recovered from MCF7 cell extracts . Cyclins A, D1, D3 and E , Cdk 2 cdk 4, p21, p27 and p53 coeluted with DNA polymerase δ catalytic subunit, confirming the immunoprecipitation data that pol δ is associated with cell cycle proteins.

Additional work also showed that a histone kinase activity was associated with the highly purified pol δ preparations. This histone kinase activity phosphorylated both the p125 and p50 subunits on overnight incubation with [γ ^{32}P] ATP. The labeled p125 band was eluted from the gels and subjected to phosphoamino acid analysis by 2D separation on thin-layer cellulose plates. The results showed that the labeled p125 contained only phosphothreonine and phosphoserine.

This work was repeated with MCF10A, MCF7 and MDA MB231 cells. MCF10A is a phenotypically normal, nontransformed line. The cells are a spontaneously immortalized line from a culture of human breast epithelial cells from a reduction mammoplasty. The cells are diploid and possess the characteristics of normal breast epithelium. MCF7 is a malignant human breast epitheial cell line that has been widely used for in vitro mechanistic studies. MDA-MB-231 is from an adenocarcinoma , pleural effusion breast.

Thus our studies showed that 1) we can isolate an active form of DNA polymerase δ multiprotein complex from breast cancer cells by immunoaffinity chromatography and gel filtration. 2) Immunoprecipitation and Western blot analysis demonstrated the presence of cell cycle proteins providing a possible ink between cell cycle regulation and DNA replication. 3) The presence of p53, p21 and p27 in the active DNA polymerase δ complex indicate a role for these proteins in the regulation of DNA replication or DNA repair.

We have repeated the immunoblot analysis of components of pol δ complexes isolated from our pol δ immunoaffinity column several times. It was found, for example, that cyclin D1 is only found in the phenotypically normal MCF10 A cell lines and not in the cancerous MCF7 and MDA MB231 cell lines. It has been reported by Xiong et al that the cyclin D1 gene is present in a rearranged or amplified form in 20% of all breast tumors (Xiong et al.,1993a). Thus, our biochemical data agrees with their findings. These results are surprising, but are consistent with a redistribution of PCNA from the

replication complex to the cyclin complexes as has been proposed by Xiong et al. (1993b). Sequestration of PCNA by the D cyclins could explain their absence in the immunoaffinity purified pol delta.

Task 3. Study of the response of the pol δ promoter in normal and breast cancer cell lines.

The p53 tumor suppressor is involved in cell cycle arrest, differentiation and apoptosis. It transactivates many genes that regulate cell cycle and cell growth. The DNA polymerase δ catalytic subunit gene (POLD1) was studied as a transcriptional target of p53 in response to DNA damage. Northern blot analyses showed that POLD1 steady-state mRNA was repressed by about 80% when ectopic wild-type p53 expression was induced to a physiologically relevant level in "tet-off" cultured cells in which p53 was expressed. Moreover, transfection assays demonstrated that p53 was able to repress Sp1-stimulated POLD1 promoter activity, and that this repression was largely due to the loss of the sequence specific interaction between Sp1 protein and an Sp1-binding site which overlaps the P4 p53-binding site. Finally, gel shift and co-immunoprecipitation assays suggested that p53 competes with Sp1 protein for binding to the P4 sequence of the POLD1 promoter. Thus, the POLD1 gene is shown for the first time to be a transcriptional target of p53, and the repression of the POLD1 gene could be part of the pleiotropic cellular response to p53.

This work has been published in J. Biol. Chem. Baoqing Li and Marietta Y.W. Lee " Transcriptional Regulation of the Human DNA polymerasae δ catalytic subunit Gene (POLD1) by p53 tumor suppressor and Sp1" J. Biol. Chem. 276, 29729-29739 (2001).

Technical objective 2 The multi-protein complexes of pol δ from normal and breast cancer cell lines and tissues will be studied to determine if a) they display functional defects and b) to determine if they exhibit altered behavior in terms of protein-protein interactions.

Task 4. Comparison of DNA replication functions of multi-protein pol δ complexes of normal and breast cancer cells.

Dr. Krucher showed that the product of the retinoblstoma tumor suppressor gene (pRb) interacted with the catalytic subunit of DNA polymerase δ. This work has been published "Interaction of the Retinoblastoma protein (pRB) with the catalytic subunit of DNA polymerase delta (p125) "Krucher, N., Zygmunt A., Mazloum, N.,Tamrakar, S., Ludlow, J.W. and **Lee**, M.Y.W.T..*Oncogene*, (2000) 19, 5464-5470

Task 5. Study of protein-protein interactions within complexes of pol δ .

This is well underway. Five papers resulted from this work :

1. Characterization of the p125 Subunit of Human DNA Polymeraseδ and Its Deletion Mutants" *J. Biol Chem.* 273, 9561-9569 (1998)

2. Identification of DNA Replication and Cell Cycle Proteins that Interact with PCNA. *Nucleic Acids Research* 25, 5041-5046 (1998)

3. Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA Polymerase δ. *J.Biol. Chem.* 274, 26647-26653(1999)

4. Evidence that DNA polymerase δ isolated by immunoaffinity chromatography exhibits high molecular weight characteristics and is associated with the KIAA0039 protein and RPA. *Biochemistry*, 39, 7245-7254 (2000)

5. Identification of a Fourth Subunit of Mammalian polymerase δ. *J.Biol. Chem* 275, 18739-18744 (2000)

We have developed a native gel electrophoresis technique (Jaime et al.,in prep). This coupled with PCNA overlay technique will be very powerful tool to study protein - protein interaction between pol δ and PCNA complexes.

Using yeast two hybrid analysis we have also discovered a novel protein, p38, that interacts with the p50 subunit of pol δ, and may represent a new subunit of DNA polymerase δ (Liu et al., in preparation)

Using yeast two hybrid and the second subunit of replication factor C RFC 40 as a bait a graduate student supported by this grant (Rakhee Gupte) found that 50 % of the clones encode the regulatory subunit R1α of the cAMP-dependent PKA. To provide further evidence for this interaction, she carried out co-immunoprecipitation experiments

using MCF10A and MCF 7 cells. In both of these cell lines she could show association of RFC 40 with the regulatory subunit RIα of the cAMP dependent PKA. In vitro binding assays were conducted in order to provide further evidence that RFC 40 interacts with the regulatory subunit R1α of the cAMP dependent PKA, independent of the catalytic subunit of PKA. Pull down assays were performed using bacterially expressed full length GST-RFC40 bound to glutathione beads and bacterially expressed full length His-tagged PKA-R1α. These studies confirmed a specific interaction between these two proteins. Furthermore, to determine the exact region involved in this interaction N-terminal and C-terminal deletion mutants for each of the two proteins were constructed. The N-terminus of the regulatory subunit R1α of PKA and the C terminus of RFC40 were found to be involved in protein protein interaction in an in vitro binding assay.

By using confocal microscopy, she co-localized these proteins to the nucleus in MCF 7 cells. She also used laser scanning cytometry studies to determine the subcellular distribution of each of these proteins in different phases of the cell cycle. The levels of both of these proteins increased significantly in the nucleus from G0/G1 to S to G2/M phase. On screening the amino acid sequence of R1α , she found that it may have a non-conventional nuclear localization sequence. Two mutants of GFP R1 α- one with the putative NLS but with the RFC40 binding site deleted and one with both sites deleted. Transfection experiments in 293 cells showed that the R1α mutant lacking the NLS remained in the cytosol These results support the view that we have identified for the first time the NLS for R1α. Co-localization of the endogenous RFC40 in these same experiments showed that it remained in the cytosol and that localization to the nucleus was blocked. These results show that the nuclear transport of RFC 40 requires R1α with an intact RFC 40 binding site, and are consistent with an unexpected and novel hypothesis that R1α function as a transport protein for RFC40 in addition to its traditional role as a regulator of PKA.

Technical Objective 3. The ability of extracts of the breast cancer cell lines to carry out nucleotide excision repair will be compared to that of normal breast cell lines using an in vitro repair assay.

Task 6. Assay of repair activities of nuclear extracts of normal and breast cancer cell lines.

Nayef Mazloum has been setting up the repair assays. He has been developing various templates that simulate homologous recombination. Preliminary data are quite encouraging. Pol δ has been implicated in an array of DNA repair pathways such as long patch excision repair (BER), nucleotide excision repair (NER), double stranded break repair (DSB), homologous recombination and mismatch repair. The functional integration of pol δ into these cellular processes that involve multi-protein complexes requires its physical interaction with target proteins in these complexes. In order to identify novel protein-proteins interactions involving pol δ, we employed a proteomics approach that could detect femtomole amounts of protein that could not be detected by regular techniques. We employed LC/MS/MS analysis on the tryptic digests of the pol δ complex purified by ion exchange, phenyl agarose and immuno-affinity chromatography with a monoclonal antibody against the catalytic subunit p125. An interesting array of proteins was revealed upon analysis, which could be classified into eight groups: DNA replication, DNA damage, RNA binding proteins, cell division, protein phosphatases, heat shock proteins, proteasomal proteins and hypothetical proteins. We identified an interaction between pol δ and nuclear DNA helicase II (NDHII). Functional analysis of the polymerase peak fraction revealed the association of a 3' to 5' helicase when assayed on M13mp19 plasmid annealed to a 5' labeled oligomer. Antibody arrays against cell cycle regulatory proteins confirmed some of the interactions obtained from the sequencing data and revealed new interactions between pol δ and the DNA damage sensing pathway.

Task 7. Study of repair synthesis of multi-protein pol δ complexes from normal and breast cancer cells.

Nayef Mazloum has recently initiated these studies. Preliminary data showed that a helicase stimulates the polymerase δ activity on linearized exonuclease dependent M13 templates.

Technical objective 4. Normal and breast cancer cell lines and tissues will be screened for genetic alterations in the pol δ and PCNA gene

Task 8. RT-PCR and genomic PCR analyses of 3' to 5' exonuclease and N2 domains of pol δ .

We have screened for mutations in the 3' to 5' exonuclease domains of the POLD1 gene in breast cancer tissues using a Non-Isotopic RNase Cleavage Assay (NIRCA) and DNA sequencing techniques. Four novel mutations, P327L, D445G, S478G, R507H were identified. Two of the four mutations (S478G, R507H) are within the proofreading Exo motifs at positions conserved throughout evolution. Although a crystal structure of the eukaryotic pol δ has not been obtained, we have used computer modeling based on the 3-D crystal structure of RB69 bacterial DNA polymerase. We found a ca. 40% homology in the pol δ 3' 5' exonuclease domains with those of RB69 DNA polymerase. This analysis indicates that P327L is in the DNA editing channel, which is very important in 3' 5' exonuclease activity. D445G might change the conformation of a β hairpin sheet which is supposed to switch DNA from the polymerase domain to the exonuclease active sites, thus affecting the proofreading activity of p125. The significance of these findings is that we have now obtained evidence for mutations in pol δ in breast cancer tissues which could affect the proof reading capacity of the pol δ enzyme. This would support the view that mutations in pol δ may contribute to the accumulation of mutations within breast cancer cells.

Task 9. Analysis of functional properties of mutations found in breast cancer cell lines and tissues.

In order to analyze the functional properties of mutations of the pol δ p125 subunit we have to have a recombinant expression system which will allow the assembly of the pol δ complex. This project became more complex because of our discovery that there were two additional subunit in the complex. We have just succeeded in the overexpression of the four subunits of DNA polymerase δ. The paper has been submitted to J. Biol. Chem. (2002) "Reconstitution and characterization of human DNA polymerase δ". We have generated polymerase δ p125 deletion mutants and site directed mutants in the baculovirus overexpression system based on the results from breast cancer samples. The recombinant mutants will be purified and changes in function will be characterized

.

Task 10. PCR analyses of the promoter region of pol δ in breast cancer cell lines and tissues.

Using NIRCA and DNA sequencing stated in Task 8, we are also investigating the mutants of the POLD1 promoter. We have amplified a length of 2 kb of the POLD1 genomic DNA fragment covering the full length 1.8 kb from different breast cancer tissues and different breast cancer cell lines If we find some interesting mutated sites in

the POLD1 promoter, we will introduce these mutations into the pGL2 delta reporter plasmid. The function of the mutated promoter will be compared to that of the normal promoter.

Task 11. RT-PCR and genomic PCR analyses of human PCNA.

p21 was found in the pol δ complex from the normal cell line (MCF 10A) but was absent in MDA MB 231 complexes and was lost from the MCF7 complex after gel filtration. Our conclusion is that p21 is dissociated from these complexes on gel filtration.

p21 is known to inhibit cell progression by binding to G1 cyclin-dependent kinase (CDK) complexes. p21 also binds to proliferating cell nuclear antigen (PCNA) and inhibits the ability of PCNA to activate DNA polymerase delta (pol δ), the principal replicative DNA polymerase that is involved in both DNA replication and DNA repair. Using a yeast two hybrid screening technique and the p50 subunit of DNA pol δ as a bait, we found that p21 binds to DNA polymerase δ. We confirmed the p21 and p50 interaction by using GST-p50 and GST-p21 pull down assays. We also obtained in vivo evidence of this interaction through co-immunoprecipitation in MCF7 breast cancer cells by using monoclonal antibody against p50 or polyclonal antibody against p21. We found that p21 associates with other components of DNA polymerase δ in the replication complexes by using GST-p21 pull down and by Far-Western techniques. A pairwise yeast two hybrid assay showed that the N-terminus 1-107aa of p50 and the C-terminus 83-164aa of p21 may be involved in this interaction. We conclude that p21 can bind to DNA pol δ directly. The functions of the interaction between DNA pol δ and p21 are still under studying We have also studied in detail the association between p21 and DNA polymerase delta. This work will be presented in the upcoming Era of Hope 2002 DOD Breast Cancer Research program meeting.

We have so identified a novel PCNA binding motif by panning of a random peptide display library. The work was published in Biochemistry (2001) 40, 4512-4520.

To further characterize the sites and function of PCNA interaction, we constructed nine mutants in the region adjacent to the interdomain connnector loop of PCNA by site directed mutagenesis. The mutant proteins were overexpressed in E. coli and purified to near homogeneity. Preliminary data showed that some mutants can affect DNA synthesis of DNA polymerase δ. This work is being written up for publication. (Wang et al., in preparation).

6. Key Research Accomplishments

As can be seen from the summaries given above, as well as our list of published work, we have accomplished a great deal of the work that was proposed on a broad number of fronts.

A summary of our key accomplishments during the period of support is given below.

A. Discovery of a novel mechanism for the regulation of DNA replication by p53. (Tasks 1 and 3)

We have shown that the pol δ gene message and protein levels decline after DNA damage, and that this is inversely correlated to changes in p53. We have shown for the first time that p53 transcriptionally represses the pol δ gene, and have shown in a detailed study the identity of the promoter region in the POLD1 gene that is the site for p53 regulation. This provides a novel mechanism for a linkage between DNA damage, p53 and the down regulation of the POLD1 gene.

B. Identification of mutations of the pol δ gene in breast cancer. (Task 8).

We have shown for the first time that there are mutations in the exonuclease gene in breast cancer tissues. This work indicates that the number of mutations in the pol δ are quite frequent, even though a relatively small sample of tissues was examined. Our work provides supporting evidence for a important hypothesis that mutations in the proof reading exonuclease region of pol δ may play a role in generating a mutator phenotype in breast cancer. The significance of these findings cannot be understated and provides a solid foundation for future work in this area.

C. Discovery of two new subunits of the pol δ complex (Technical objective 2).

We have discovered two new subunits of the pol δ enzyme complex, the p68 and p12 subunits, and have reconstituted the four subunit complex in the Sf9 cell system. This is a major accomplishment and a necessary step in any attempt to understand changes in the pol δ system that accompany the cancer process. In addition, two new accessory proteins, p38 and p46 were identified by the yeast two hybrid screening method.

D. Novel protein interactions in the pol δ system in normal and cancer cells.

We have used breast cancer cell lines to probe for changes in proteins associated with polymerase δ complexes isolated by immunoaffinity chromatography.

In the case of the transformed breast cell lines, significant differences were observed in that several of the protein ligands were not present in the affinity purified fraction, consistent with the findings of other investigators. Of significant note is that after immunoaffinity column and after gel filtration p21 is present in MCF10A cells but absent in MCF7 and MDAMB231 breast cancer cell lines.

We have described several novel protein protein interactions involving the pol δ system that could be potential loci for changes in the cancer process:

1) interaction between p125 and p21
2) interaction between p125 and PCNA
3) interaction between the tumor suppressor Rb and p125.
4) identification of a novel PCNA binding motif.
5) identification of the Riα subunit as a putative nuclear transport protein for the RFC40 subunit.

7. Reportable Outcomes

Manuscripts.

1. Wu S.M., Zhang, P., Zeng, X.R., Zhang, S.J., Mo, J., Li, B.Q., and **Lee,** M.Y.W.T.
   Characterization of the p125 subunit of Human DNA polymerase δ and Its Deletion Mutants". J. Biol Chem 273, 9561-9569 (1998)
2. Loor, G., Zhang, S.J., Zhang, P., Toomey, N.L., and **Lee**, M.Y.W.T.
   Identification of DNA replication and cell cycle proteins that interact with PCNA *Nucleic Acids Research* 25, 5041-5046 (1998)
3. Zhang, P., Mo, J., Perez, A., Leon, A., Liu, L., Mazloum, N., Xu, H., and **Lee,** M.Y.W.T.
   Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA polymeraseδ.
   J.Biol. Chem. 274, 26647-26653(1999)
4. Mo, J., Liu, Li, Leon, A., Mazloum, N., and **Lee**, M.Y.W.T.
   Evidence that DNA polymerase δ isolated by immunoaffinity chromatography exhibits high molecular Weight characteristics and is associated with the KIAA0039 protein and RPA.

Biochemistry , 7245-725439 (2000)

5. Liu, L., Mo, J., Belmonte, E., and **Lee**, M.Y.W.T.
   Identification of a Fourth Subunit of Mammalian polymerase δ.
   J. Biol Chem. 275, 18739-18744 (2000)
6. Xu, H., Zhang, P., Liu, L., and Lee, M.Y.W.T.
   A Novel PCNA-binding motif identified by the panning of a random peptide display library.
   Biochemistry 40, 4512-4520 (2001)
7. Liu, Li., Belmonte, E., Mazloum, N., Xu, H., and **Lee**, M.Y.W.T.
   Cloning and characterization of a novel protein p38 which interacts with DNA polymerase δ and p50 small subunit .
   Submitted to J. Biol. Chem. (2002)
8. Xu, H., Zhang, P., Mazloum, N., and **Lee**, M.Y.W.T.
   Mutational Analysis of the Exo Motif of POLD1 gene in human Breast Cancer cells
   (in preparation)
9. Jaime, C., Mazloum N., and **Lee**, M.Y.W. T. Isolation of proteins associated with DNA polymerase δ using an immunoaffinity column.
   (In preparation)
10. Wang, J., Weng Y., and Lee, M.Y.W. T. Determination and characterization of Interaction Sites between human PCNA and human DNA polymerase δ .
    (in preparation)

Abstracts.

1. Li, B.Q., Chen, X., and **Lee**, M.Y.W.T. Transcriptional repression of DNA polymerase delta catalytic subunit Gene (POLD1) by p53 tumor suppressor. Abstract #443 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999
2. Liu, L., Rodriquez-Belmonte, E., and **Lee**, M.Y.W.T. Cloning the putative human DNA polymerase δd thilrd subunit, which binds to the small subunit p50 and PCNA Abstract #337 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999
3. Mazloum, N., **Lee**, MY.W.T. , and Zhang, P. The Examinatin of protein-protein interaction between the catalytic subunit of DNA polymerase δ (p125) and the proliferating cell nuclear Antigen (PCNA) Abstract #343 _mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

4. Mazloum , N., Krucher, N., Liu, L., and **Lee**, M.Y.W.T. Physical Association between DNA polymerase δ and P21. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999
5. Liu, L., Rodriquez-Belmonte, E., and **Lee**, M.Y.W.T. DNA polymeraseδ holoenzyme: how many subunits are there? Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999
6. Gupte, R., Mo, J., Liu, L., Mazloum, N., and **Lee**, M.Y.W.T. Further Studies of the Immunoaffinity purified Pol delta complex Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999
7. Zhang, P., Liu, L., Mo, J., Mazloum, N., Xu, H., and **Lee**, M.Y.W.T. Direct Interaction of PCNA with p125, the catalytic subunit of mammalian DNA polymerase delta. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999
8. Xu, H., and **Lee**, M.Y.W.T. Analyzes of POLD1 gene mutation and study of its transcriptional regulation in Breast Cancer Cells. Breast Cancer Research Program Era of Hope 2000 p391
9. Rahmeh, A., Li, B., Jaime, C., Mazloum, N., and Lee, M.Y.W. T. The Tumor Suppressor Protein p53 interacts with DNA poymerase delta at the transcriptional level as well as at the protein level: a possible role for p53 in regulating DNA polymerase delta dependent replication and repair Pathways. Cold Spring Harbor Meeting on Eukaryotic DNA replication (2001) p188
10. Li, H., Liu, L., Xie, B., Mazloum, N., and Lee, M.Y.W. T. p21 interacts with the p50 subunit of DNA polymerase delta. Cold Spring Harbor Meeting on Eukaryotic DNA replication (2001) p148
11. Liu, L., Belmonte, E.M. , Xu, H., Mazloum, N., and Lee, M.Y.W. T. Cloning and Characterization of a novel protein p38 which interacts with Dna polymerase delta and PCNA. Cold Spring Harbor Meeting on Eukaryotic DNA replication (2001) p152
12 Xie, B., Mazloum, Wang,J., Liu, L., and Lee, M.Y.W. T. Reconstitution of human DNA polymerase delta using the baculovirus overexpression system. Cold Spring Harbor Meeting on Eukaryotic DNA replication (2001) p. 224
13. Gupte, R., Liu, L., and Lee, M.Y.W. T. Protein-prpotein interaction between human Replication Factor C and regulatory subunit R1 alpha of PKA. . Cold Spring Harbor Meeting on Eukaryotic DNA replication (2001) p. 89
14. Li, H., Liu, L., Xie, B., and Lee, M.Y.W. T. Association between p21 and DNA polymerase delta. Breast Cancer Research Program Era of Hope 2002

Oral Presentations

1. Liu, L., Rodriquez-Belmonte, E., and **Lee**, M.Y.W.T. Cloning the putative human DNA polymerase δ third subunit, which binds to the small subunit p50 and PCNA Abstract #337 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999
2. Zhang, P., Liu, L., Mo, J., Mazloum, N., Xu, H., and **Lee**, M.Y.W.T. Direct Interaction of PCNA with p125, the catalytic subunit of mammalian DNA polymerase δ. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999.

Degrees Obtained:

1. Li Liu Ph.D Dissertation Thesis "Cloning and Characterization of Novel Proteins which associate with Mammalian Dna Polymerase δ" 1999
2. Baoqing Li Ph.D Dissertation Thesis" Transcriptional Regulation of Human DNA polymerase δ catalytic subunit gene (POLD1) by p53 and Sp1" 2000
3. Nayef Mazloum. Nayef is working on his thesis "Tandem Mass Spectrometry Analysis of DNA Polymerase Delta Complex. Insights on the Regulation of DNAreplication repair and recombination Pathways. As discussed in tasks 6 and 7 , he has developed several repair assays and will continue these work in his thesis. He will eventuallly enter Medical School and become an oncologist with the hope of continuing cancer research.
4. Rakhee Gupte. Ms Gupte will defend her thesis on "Protein-protein interaction between human Replication Factor C and regulatory subunit R1 alpha of PKA". early this summer.

Employment opportunities received on experiences/training supported by this award

1. Li Liu	DOD postdoctoral fellow, New York Medical College
2. Baoqing Li	Postdoctoral fellow, Columbia University College of Physicians and Surgeons. As of 2001, Medical Student. New York Medical College. Dr. Li wants to specialize in Oncology and Cancer.
3. Nancy Krucher	Assistant Professor, Department of Biological Sciences, Pace University , Pleasantville, New York. She b\obtained her own R15 from NIH as of this month.
4. Peng Zhang	Senior fellow, Scientist. Division of Experimental Therapeutics. Walter Reed Army Institute of Research Silver Spring ,MD 20910 Dr. Zhang is writing his own grant as of last month.
5. Cindy Jamine	Cindy is now a third year medical student at UCLA.

6. Jining Wang	Jining is now a research associate at Stanford University.
7. Hao Li	Philip Morris postdoctoral fellow

8. Conclusions.

DNA polymerase δ, the principal replicative DNA polymerase in mammalian system is responsible for the elongation of leading strand and for the completion of Okazaki fragment on lagging strand synthesis. We have used an immunoaffinity column to the p125 subunit and co-immunoprecipitation experiments to show that pol δ is linked to cell cycle and repair enzymes. We have linked polymerase δ to p21, and to the tumor suppressor genes p53 and Rb. These are of significance for the finding that p53 transciptionally represses polymerase δ is a novel route for its regulation of DNA replication after being induced by DNA damaging agents. Mutational analyses of the exonuclease regions of POLD1 gene in human breast cancer cell lines and tissues led to the finding of mutations that support the hypothesis that pol δ may contribute to the mutator phenotype during tumor development.

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Characterization of the p125 Subunit of Human DNA Polymerase δ and Its Deletion Mutants

INTERACTION WITH CYCLIN-DEPENDENT KINASE-CYCLINS*

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The catalytic subunit of human DNA polymerase (pol) δ was overexpressed in an active, soluble form by the use of a baculovirus system in insect cells. The recombinant enzyme was separated from endogenous DNA polymerases by phosphocellulose, Mono Q-Sepharose, and single-stranded DNA-cellulose chromatography. Recombinant DNA pol δ was also purified by immunoaffinity chromatography. The enzymatic properties of the purified catalytic subunit were characterized. The enzyme was active and possessed both DNA polymerase and associated 3′ to 5′ exonuclease activities. NH_2-terminal deletion mutants retained polymerase activity, whereas the core and COOH-terminal deletion mutants were devoid of any measurable activities. Coinfection of Sf9 cells with recombinant baculovirus vectors for pol δ and cyclin-dependent kinase (cdk)-cyclins followed by metabolic labeling with $^{32}P_i$ showed that the recombinant catalytic subunit of pol δ could be hyperphosphorylated by G_1 phase-specific cdk-cyclins. When cdk2 was coexpressed with pol δ in Sf9 cells, pol δ was found to coimmunoprecipitate with antibodies against cdk2. Experiments with deletion mutants of pol δ showed that the NH_2-terminal region was essential for this interaction. Coimmunoprecipitation and Western blot experiments in Molt 4 cells confirmed the interaction *in vivo*. Preliminary experiments showed that phosphorylation of the catalytic subunit of pol δ by cdk2-cyclins had little or no effect on the specific activity of the enzyme.

DNA polymerase (pol)[1] δ is the central enzyme in eukaryotic DNA replication (1) and also serves an important role in DNA repair (2). Isolation of the calf thymus (3) and human (4) enzymes has shown that it consists of at least two core subunits of 125 and 50 kDa. The hallmarks of this polymerase are that it has an intrinsic 3′ to 5′ exonuclease activity, distinguishing it from pol α and pol β. The 125-kDa subunit of human pol δ (p125) has been identified as the catalytic subunit (4). Pol δ is a member of a family of DNA polymerases which includes DNA polymerase α, pol ε, the herpesvirus DNA polymerases, and bacteriophage T4 polymerase (5, 6). Examination of the regions of conserved sequence has led to the identification of domains that are potentially required for DNA interaction, deoxynucleotide interaction, as well as the 3′ to 5′ exonuclease activity of pol δ (7). In addition, there are several regions in the NH_2 and COOH termini which are conserved among human pol δ, yeast pol δ, and yeast and human pol ε (5, 7).

Studies of the replication of SV40 DNA *in vitro* have led to the identification of a number of accessory proteins, which, together with pol δ, are required for the formation of a replication complex at the replication fork. These include PCNA, which functions as a sliding clamp and enhances the processivity of pol δ, consistent with its role as the leading strand polymerase (8). Although there have been some mutagenesis studies of the yeast pol δ (9), little has been done with human or mammalian pol δ, largely because of the lack of a suitable expression system. To facilitate structure-function studies of pol δ, it is desirable to have an expression system for the production of the recombinant protein. The expression of the human pol δ catalytic subunit has been achieved in mammalian cells using a vaccinia virus vector (10). In this study we report the expression of p125 in Sf9 cells using a baculovirus vector as well as methods for separating the recombinant protein from endogenous DNA polymerases in baculovirus-infected Sf9 cells. Deletion mutants of p125 were also characterized to investigate the domain structure of pol δ. In addition, we have obtained novel evidence that pol δ p125 is phosphorylated by the cyclin-dependent kinase (cdk)-cyclin complexes and also can be coimmunoprecipitated with cdk2 when they are coexpressed in Sf9 insect cells. The interaction of pol δ with the cyclins and cdks was also confirmed by coimmunoprecipitation and Western blot experiments in Molt 4 cells. Preliminary experiments showed that phosphorylation has moderate or little effect on the activity of the catalytic subunit.

EXPERIMENTAL PROCEDURES

Materials—Sf9 cells were purchased from Invitrogen and were maintained at 27 °C in TNM-FH insect medium supplemented with 10% fetal calf serum and 50 μg/ml gentamycin. Cells were propagated both as adherent monolayers and as nonadherent suspension cultures. These cells were used as the hosts for the propagation of wild type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and recombinant baculoviruses. Cyclin and cdk recombinant baculoviruses were gifts of Dr. Charles Sherr (St. Jude's Hospital, Memphis, TN). BaculoGold™-linearized baculovirus DNA was purchased from Pharmingen. The baculovirus transfer vector P2bac was purchased from Invitrogen. Plasmid pALTER-1 was purchased from Promega.

Construction and Screening of Recombinant Baculoviruses—The coding sequence of pol δ which was used in these studies was derived from the cDNA originally isolated by Yang *et al.* (7). This coding sequence was inserted into the pALTER vector and corrected by site-directed

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[1] The abbreviations used are: pol, polymerase; p125, 125-kDa subunit of human pol δ; cdk, cyclin-dependent kinase; AcMNPV, *A. californica* multiple nuclear polyhedrosis virus; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen.

mutagenesis so that His-119, Asn-173, and Gly-776 were mutated to Arg-119, Ser-173, and Arg-776 to conform to the genomic sequence (10, 11). The plasmid pALTER-pol δ containing the corrected full-length pol δ coding sequence (3.5 kilobases) was excised from the pALTER plasmid by *Bam*HI/*Hin*dIII digestion, gel purified, and inserted into *Bam*HI/*Hin*dIII-digested baculovirus transfer vector p2bac. The recombinant p2bac plasmids were cotransfected into Sf9 cells with wild type baculovirus DNA according to Ausubel *et al.* (12). Wild type BaculoGold™-linearized AcMNPV DNA (1 μg), recombinant plasmid DNA (3 μg), cationic liposome solution (25 μl), and 1 ml of Grace's insect medium containing no supplements were mixed by vortexing for 10–15 s and incubated at room temperature for 15 min. The transfection mixture was then layered onto Sf9 cells growing on 60-mm plates. After 4 days at 27 °C, the medium was aspirated and analyzed for virus production by plaque assay. The recombinant baculoviruses were identified as occlusion-negative plaques with a dissecting microscope. Because the BaculoGold™-linearized virus DNA contains a lethal deletion and a *lacZ* gene, the small portion of nonrecombinant virus plaques stained blue on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside plates, whereas all recombinants produced colorless plaques on these plates. After three rounds of plaque purification, pure recombinant baculoviruses were obtained. Occlusion-negative viral stocks were prepared from the final supernatants, titered, and stored at 4 °C. Deletion mutants of pol δ were constructed as described in Ref. 13.

Infection of Sf9 Cells with Recombinant Baculovirus and Preparation of Cell Extracts—Recombinant viral stocks (0.5 ml) were added to a multiplicity of infection between 5 and 10 for the infection of log phase Sf9 cells for 1 h. The inoculum was then removed from the plates, and 8 ml of fresh complete TNM-FH insect medium was added. The infected Sf9 cells were allowed to grow for 2 days at 27 °C and were harvested 48 h postinfection. Cells were harvested from 80 100-mm plates and collected by centrifugation. The cell pellets were washed twice with ice-cold phosphate-buffered saline, pH 7.4. Subsequent manipulations were carried out at 4 °C. The cells from 80 plates (about 8×10^8 cells) were suspended in 5-cell pellet volumes (50 ml) of lysis buffer (40 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 0.1 M NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine-HCl). Cells were disrupted by passage through a French press at 1,000 p.s.i. The lysate was centrifuged at $27,000 \times g$ for 30 min. The supernatant was removed and saved as the soluble extract, and the pellet was suspended in 20 ml of lysis buffer plus 0.5 M NaCl and sonicated three times for 20 s each at 50 watts on ice. The extract was again centrifuged at $27,000 \times g$ for 30 min, and the supernatant was designated as the high salt-solubilized fraction. Protein concentrations of the first and second extracts were 12 and 9 mg/ml, respectively. The pellet was then dissolved in 1 ml of 8 M urea. The two fractions (low and high salt extracts) were then combined and dialyzed against TGEED buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, I mM dithiothreitol).

Phosphocellulose Chromatography—The dialyzed lysates were loaded onto a phosphocellulose column (5 × 7 cm) equilibrated in TGEED buffer. The column was eluted with a linear gradient of 50–1 M NaCl in TGEED buffer in a total volume of 2 liters. Fractions of 10 ml each were collected and assayed for DNA polymerase activity. Western blots were also performed using 38B5, a monoclonal antibody against the COOH-terminal region of pol δ (2, 14).

HPLC—The combined fractions from the phosphocellulose column which contained recombinant pol δ p125 were dialyzed against TGEED buffer, pH 7.8, passed through an 0.45-μm syringe filter, and injected onto a Mono Q HR 5/5 column. The enzyme was eluted with a linear gradient of 0–1 M NaCl for 20 min at 1 ml/min.

Single-stranded DNA-cellulose Chromatography—Fractions from the Mono Q column were dialyzed against HEPES buffer (20 mM HEPES, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5) and were then loaded onto a single-stranded DNA-cellulose column (0.5 × 6 cm) equilibrated with HEPES buffer. The column was washed with the same buffer, and a gradient of 50–500 mM NaCl in a total volume of 40 ml was applied. Fractions of 1 ml were collected and analyzed by SDS-PAGE, Western blotting, and assays for pol δ activity.

Immunoaffinity Chromatography—Monoclonal antibody 78F5 was coupled onto AvidChrom hydrazide (Sigma) as described by Jiang *et al.* (14). The column (1 × 10 cm) was equilibrated with TGEE buffer (50 mM Tris-HCl, 0.1 mM EGTA, 0.5 mM EDTA, 10% glycerol, pH 7.8). The column was washed with the same buffer containing 50 mM NaCl, and pol δ was eluted with 0.2 M NaCl in TGEE buffer. Fractions of 1 ml were collected and analyzed as described above.

DNA Polymerase Assays—Sparsely primed poly(dA)·oligo(dT) was used as the template as described by Lee *et al.* (3). The standard reaction for the poly(dA)·oligo(dT) assay contained 0.25 optical density units/ml poly(dA)·oligo(dT) (20:1), 200 μg/ml bovine serum albumin, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES, pH 6.0, 100 cpm/pmol [^{3}H]TTP, and 0.2–0.4 unit of pol δ in the presence or absence of 0.2 μg of PCNA in a total volume of 100 μl. Reaction mixtures were incubated for 60 min at 37 °C and were terminated by spotting onto DE81 papers that were then washed four times with 0.3 M ammonium formate, pH 7.8, once with 95% ethanol, and counted as described previously (4).

Assay for 3′ to 5′ Exonuclease Activity—The assay was performed by measuring the release of [^{3}H]dTMP from [^{3}H]dT_{50} as described previously (3). The assay contained 2 μM [^{3}H]dT_{50} (200–300 cpm/pmol), 25 mM HEPES buffer, pH 7.4, 5 μg of bovine serum albumin, 5 mM $MgCl_2$, and 0.2–0.4 unit of pol δ in a total volume of 60 μl. Reaction mixtures were incubated for 30 min at 37 °C and were terminated by spotting 20 μl onto DE81 filter papers. Filters were washed four times with 0.3 M ammonium formate, pH 7.8, and once with 95% ethanol and counted as described previously (3).

Western Blot Analysis—The recombinant proteins expressed in Sf9 cells infected with recombinant baculoviruses were analyzed by Western blotting with pol δ monoclonal antibody 38B5 (2, 14). Extracts of Sf9 cells prepared as described above were subjected to SDS-PAGE in 5–15% gradient gels that were then transferred to nitrocellulose membranes. Prestained protein standards (Sigma) were used as molecular weight markers and also to provide visual confirmation of efficient transfer. The nitrocellulose blots were blocked with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, for 1 h at room temperature. The blots were then incubated with monoclonal antibody against pol δ for 12 h at 25 °C. After three 10-min washes in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, the blots were incubated with biotinylated sheep anti-mouse immunoglobulin for 1 h at 27 °C followed by incubation with streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide.

Coinfection of Sf9 Cells with Pol δ, Cyclins, and Cdks and $^{32}P_i$ Labeling—Sf9 cells (10^7) were grown to exponential stage. Pol δ, cyclin, and cdk recombinant baculoviruses (0.5 ml) were added as indicated. The cells were infected at room temperature for 1 h. The recombinant baculoviruses were removed, replaced with growth medium, and the cells were grown for an additional 2 days at 27 °C before labeling with $^{32}P_i$. Infected Sf9 cells were transferred into a 15-ml tube for $^{32}P_i$ labeling. After centrifugation and removal of growth medium, the cells were resuspended in 2 ml of fresh phosphate-free medium containing 200 μCi of $^{32}P_i$ (specific activity 3,000 Ci/mmol) and incubated at 37 °C for 2 h. The cells were centrifuged at $3,000 \times g$ for 5 min. The supernatant was removed, and the cells were washed twice with phosphate-buffered saline. The cells were sonicated for 30 s in 40 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 0.5 M NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine-HCl. The crude cell extracts were transferred to microtubes and centrifuged at $15,000 \times g$ for 30 min. About 20 mg of total protein was used for immunoprecipitation in the presence of 20 μg of 78F5 pol δ monoclonal antibody (2, 14) and 40 μl of protein A-Sepharose slurry at 4 °C overnight. The Sepharose beads were washed twice with sonication buffer and boiled for 5 min in 50 μl of SDS sample buffer. The proteins released from the beads were then subjected to SDS-PAGE and autoradiography.

Immunoprecipitation and immunoblotting of Molt 4 Cells with Pol δ and Members of the Cyclin and Cdk—4×10^7 exponentially growing Molt 4 cells were prepared and lysed with 300 μl of Nonidet P-40 buffer (50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 1% Nonidet P-40). The lysates were precleaned with protein A beads (50 μl of a 10% suspension) by rotating at 4 °C for 30 min. The supernatants were removed by centrifugation and transferred to a fresh tube. The antibody used for immunoprecipitation was then added in the presence of 50 μl of fresh protein A beads and incubated at 4 °C for 1 h. Anti-pol δ monoclonal antibody (20 μg), PCNA monoclonal antibody (20 μg), anti-cyclin E and A antibodies (100 μl of hybridoma cell supernatant), and anti-cdk2 polyclonal antibody (2 μl) were used for the experiments. The extracts were then centrifuged and washed with Nonidet P-40 buffer three times. After SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane and Western blotted with antibodies to cdk2, cdk5, or pol δ.

RESULTS

Expression of Pol δ p125—The expression of human pol δ in Sf9 cells infected with recombinant baculovirus was analyzed

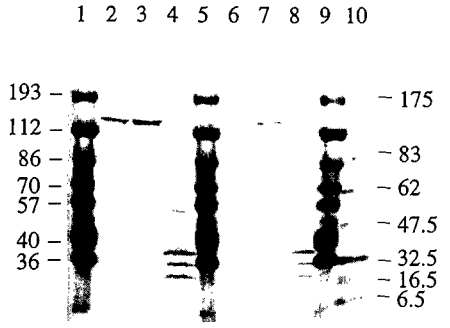

FIG. 1. **Immunoblot of extracts of Sf9 cells infected with recombinant baculovirus.** Extracts of Sf9 cells infected with recombinant baculoviruses were prepared as described under "Experimental Procedures." The cells were disrupted and extracted in 50 ml of lysis buffer containing 0.1 M NaCl and then with 20 ml of lysis buffer containing 0.5 M NaCl. The pellet was then dissolved in 1 ml of 8 M urea. These three extracts (60 or 30 μg of protein/*lane*) were then analyzed by SDS-PAGE (5–15% acrylamide). Western blotting was performed using monoclonal antibody 38B5 against human pol δ. *Lanes 1*, *5*, and *9* are high molecular weight standards as marked; *lanes 2–4* are 60 μg of the 0.1 M NaCl, 0.5 M NaCl, and 8 M urea extracts, respectively. *Lanes 6–8*, same as *lanes 2–4* but with 30 μg of protein/lane; *lane 10*, low molecular weight protein standards as marked.

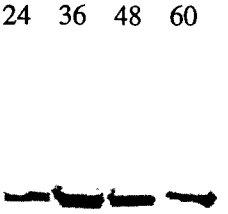

FIG. 2. **Time course of pol δ expression in Sf9 cells.** Sf9 cells infected with recombinant virus were harvested at 12, 24, 36, 48, and 60 h after infection. The cells were lysed and extracted as described under "Experimental Procedures." The 0.1 M and 0.5 M NaCl extracts were combined and analyzed for the expression of pol δ by SDS-PAGE (20 μl/*lane*) followed by immunoblotting. *Lanes* are marked according to time of harvest.

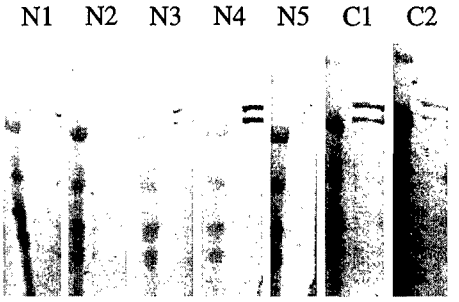

FIG. 3. **Immunoblot of crude recombinant pol δ extract using peptide-specific antibodies.** Sf9 cells were infected with recombinant baculovirus, and the cell extracts were immunoblotted using polyclonal antibodies against specific peptides derived from the NH_2- and COOH-terminal regions of the pol δ sequence (13). These were as follows: N1 (84–101), N2 (129–149), N3 (244–262), N4 (276–295), N5 (312–331), C1 (1047–1068), and C2 (1069–1090). The figure shows a composite of individual blots, each of which shows two *lanes*, the *left lane* being the prestained protein standards and the *right lane*, the Sf9 cell extracts (20 μl, 50 μg of protein).

by immunoblotting with a pol δ monoclonal antibody (38B5; see "Experimental Procedures"). The infected cells were disrupted by passage through a French press in 0.1 M KCl and centrifuged to provide the first extract. The pellet was reextracted by sonication in 0.5 M KCl (second extract). The pellet was then dissolved in l ml of 8 M urea. Immunoreactive protein was found to be present in the two salt extracts but not in the urea extract when equal amounts of protein were loaded from each fraction (Fig. 1). These experiments showed that pol δ was expressed as a soluble protein that can be extracted completely by 0.5 M KCl. Immunoblots of the corresponding extracts of Sf9 cells infected with wild type AcMNPV using the same antibody showed the absence of immunoreactive polypeptide (not shown). The time course of pol δ expression was examined by immunoblot analysis of cells taken at intervals after infection with recombinant virus (Fig. 2). For these experiments the 0.1 and 0.5 M KCl extracts were combined. Very little p125 immunoreactivity was observed at 12 h postinfection, and the peak of expression was found to be between 36 and 48 h (Fig. 2).

The recombinant pol δ was immunoblotted using a series of peptide-specific antibodies (Fig. 3) as described by Hao *et al.* (5). The different peptide-specific antibodies (N1, N2, N3, N4, N5, C1, and C2) recognized the recombinant p125 expressed in the baculovirus system. This experiment provided additional confirmation of the identity of the overexpressed protein. Note that the immunoblots (Fig. 3) for p125 appear as a doublet. As we will show, p125 could be purified to a single polypeptide of 125 kDa, although it was often observed as a doublet. A similar behavior was encountered in the isolation of the calf thymus enzyme. At present the most likely explanations are that this may reflect posttranslational modification of the enzyme by phosphorylation or partial proteolysis.

Purification of Recombinant Pol δ—Cells from 80 100-mm plates of Sf9 cells infected with recombinant baculovirus were harvested as described under "Experimental Procedures." A potential complication for the isolation of the recombinant human pol δ from Sf9 is the presence of endogenous DNA polymerases (15), which could compromise studies of the enzymatic properties of human recombinant pol δ. We have circumvented this by passing the crude extract through a phosphocellulose column ("Experimental Procedures"). When the crude extract was chromatographed on a phosphocellulose column, two peaks of activity were detected using poly(dA)·oligo(dT) as a template. One peak eluted at about 0.4 M NaCl and the second at 0.6–0.7 M NaCl (Fig. 4, *center panel*). To determine which of the peaks was the overexpressed pol δ, immunoblots were performed using monoclonal antibody 38B5. Only the first peak of activity (fractions 80–120) was immunoblotted; the second peak (fractions 120–160) did not contain immunoreactive protein (Fig. 4, *top panel*). The second peak also corresponded to the peak of polymerase activity eluted at about 0.7 M KCl when extracts of Sf9 cells infected with wild type AcMNPV baculovirus were chromatographed (Fig. 4, *bottom panel*). DNA polymerase δ isolated from the calf thymus was reported to elute between 235 and 320 mM KCl (3). The second peak was presumed to be endogenous DNA polymerase in baculovirus-infected Sf9 cells, which has been reported to elute from phosphocellulose at high salt concentrations (15).

The peak fractions that immunoblotted with pol δ antibody were pooled, dialyzed, and chromatographed on a Mono Q HPLC column. The column was eluted with a salt gradient as described under "Experimental Procedures" (Fig. 5). Assay of the fractions revealed a peak of DNA polymerase activity which eluted at about 350 mM NaCl. Calf thymus DNA pol δ elutes at 260 mM KCl under the same conditions (3, 4). The preparation contained a 125-kDa polypeptide that was immunoblotted by antibody 38B5 (Fig. 5, *inset*). The recombinant p125 was puri-

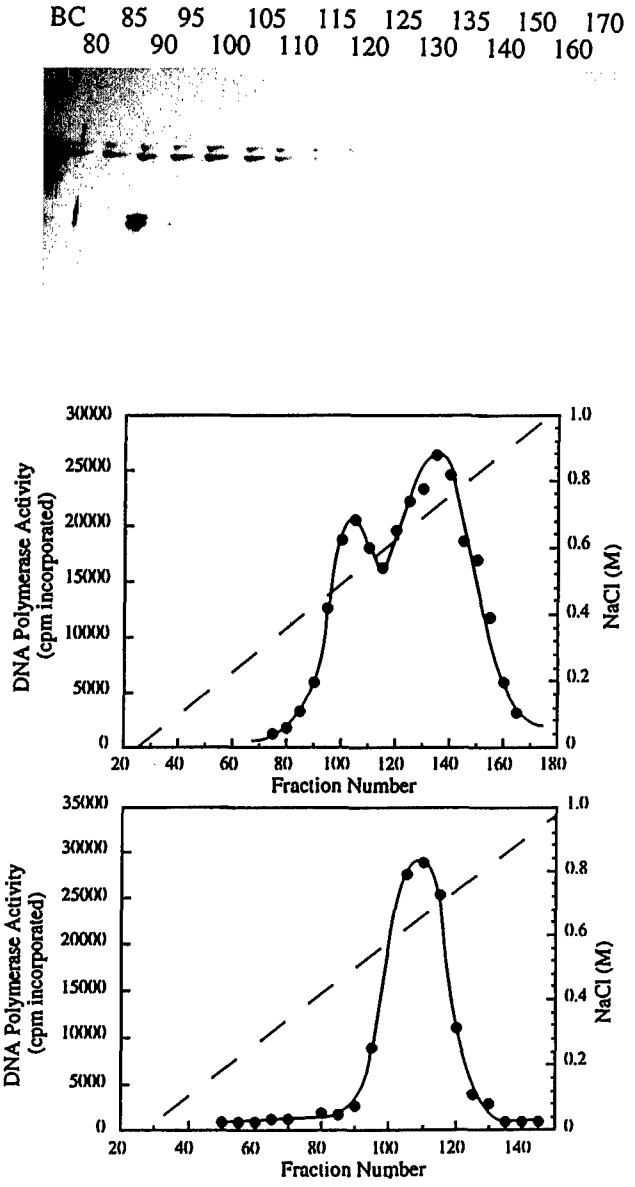

FIG. 4. **Phosphocellulose chromatography of Sf9 cell extracts infected with recombinant baculovirus.** A cell extract from Sf9 cells infected with recombinant baculovirus was chromatographed on phosphocellulose as described under "Experimental Procedures." The fractions were assayed for DNA polymerase activity using poly(dA)·oligo(dT) as template (*center panel*). The fractions containing the two peaks of activity (80–170) were immunoblotted using an antibody against pol δ (38B5) as shown in the *top panel*. *BC* refers to the extract before chromatography. A cell extract from Sf9 cells infected with the control baculovirus was also chromatographed on phosphocellulose, and the fractions were assayed for DNA polymerase activity as shown in the *bottom panel*. Immunoblots of the peak fractions failed to show any immunoreactive protein (not shown).

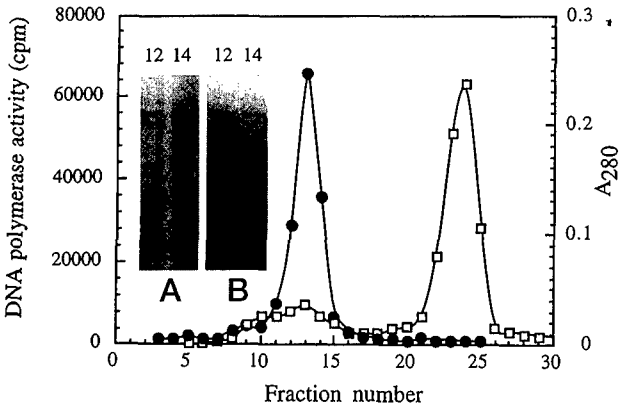

FIG. 5. **Mono Q chromatography of recombinant pol δ.** The peak fractions from the phosphocellulose chromatography step were combined and subjected to HPLC on a Mono Q 5/5 column (see "Experimental Procedures"). The enzyme was eluted with a linear gradient of 0–1 M NaCl in 20 min at 1 ml/min. The fractions were assayed for DNA polymerase activity (*closed circles*). The elution of protein is shown by the absorbance at 280 nm (*squares*). The *inset* shows the SDS-PAGE of fractions 12 and 14, which were stained for protein (*left panel*) and immunoblotted using a monoclonal antibody against pol δ (*right panel*).

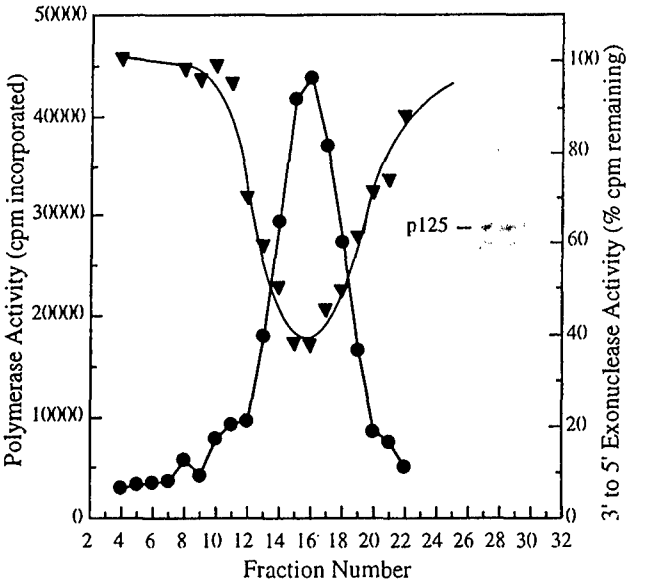

FIG. 6. **Single-stranded DNA-cellulose chromatography.** The fractions from the peak of the Mono Q column which immunoblotted with the pol δ antibody were combined, dialyzed against buffer, and loaded onto a single-stranded DNA-cellulose column as described under "Experimental Procedures." Fractions of 1 ml were collected and assayed for DNA polymerase activity (*circles*) and for exonuclease activity (*inverted triangles*). The *inset* shows the SDS-PAGE of fraction 16, which was stained for protein.

fied to near homogeneity by passage through a single-stranded DNA-cellulose column ("Experimental Procedures"). DNA polymerase activity and exonuclease activities were assayed and found to coelute (Fig. 6). The enzyme was found to be nearly homogeneous as shown by Coomassie Blue staining of SDS-PAGE of the peak fraction (Fig. 6, *inset*).

Immunoaffinity Purification of Recombinant Pol δ—We have shown previously that calf thymus pol δ can be isolated by immunoaffinity chromatography using monoclonal antibody 78F5 coupled to AvidChrom hydrazide (14). Crude Sf9 cell extracts were chromatographed on a pol δ immunoaffinity column ("Experimental Procedures"). The column was washed with buffer containing 50 mM NaCl, and pol δ was eluted by 0.2 M NaCl as shown by analysis for DNA polymerase and exonuclease activities (Fig. 7*A*) and Western blotting (Fig. 7*A*, *inset*). The enzyme obtained was still impure (Fig. 7*A*, *inset*) as determined by SDS-PAGE gels stained for protein. Sf9 cells infected with wild type virus were also passed through this immunoaffinity column, and no detectable DNA polymerase activity was recovered (Fig. 7*A*). This demonstrated that DNA polymerase activities from the Sf9 cells infected with wild type virus did not bind to the column. Note that the overexpressed p125 catalytic subunit could be eluted from the immunoaffinity column by simply using 0.2 M KCl, whereas calf thymus DNA pol δ holoenzyme is eluted at 0.4 M NaCl and 30% ethylene glycol (14). The peak fractions were combined and rechromatographed on the same column. This allowed for the isolation of

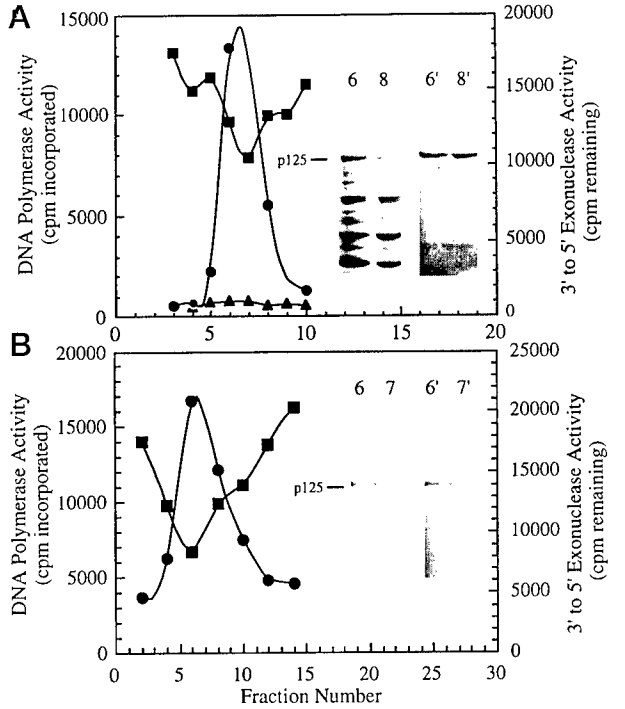

FIG. 7. **Immunoaffinity chromatography of recombinant pol δ.** *Panel A*, an extract from cells infected with recombinant baculovirus was chromatographed on a pol δ immunoaffinity column as described under "Experimental Procedures." The column was eluted with 50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, and 200 mM NaCl. Fractions of 1 ml were collected. The fractions were assayed for DNA polymerase activity (*solid circles*) and for 3′ to 5′ exonuclease activity (*solid squares*). The *inset* shows the SDS-PAGE of fractions 6 and 8 stained for protein with Coomassie Blue. The same fractions were immunoblotted using an antibody against pol δ (*lanes 6′* and *8′*). An extract from cells infected with control baculovirus was also chromatographed on the same column and assayed for DNA polymerase activity (*solid triangles*). *Panel B*, the active fractions from the first immunoaffinity chromatography (*panel A*) were pooled, dialyzed against the equilibration buffer, and rechromatographed on the same column. DNA polymerase and exonuclease activities were assayed as in *panel A*. The *inset* shows the SDS-PAGE of the peak fractions stained for protein and also immunoblotted using an antibody against pol δ.

the recombinant p125 in a nearly homogeneous form (Fig. 7*B*). Starting with 800 mg of total protein in the crude extract, about 0.11 mg of nearly homogeneous protein was recovered, presenting a purification of 153-fold and a final specific activity of 1,200 units/mg of protein using poly(dA)·oligo(dT) as a template (Table I).

Characterization of Recombinant p125—The enzymatic properties of the recombinant pol δ catalytic subunit were compared with those of native calf thymus pol δ, which had been isolated by immunoaffinity chromatography (14), and with the endogenous DNA polymerase activity from Sf9 cells infected with wild type AcMNPV (Fig. 8). The latter was the partially purified preparation obtained after phosphocellulose chromatography (see Fig. 4, *bottom panel*). The activities of the recombinant pol δ catalytic subunit were similar to those of native pol δ and the Sf9 polymerases in that they were inhibited by aphidicolin (Fig. 8*A*) and resistant to 2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate (not shown). A well known characteristic of calf thymus pol δ is its sensitivity to inhibition by *N*-ethylmaleimide; recombinant pol δ was inhibited in a manner similar to calf thymus pol δ, whereas the Sf9 polymerase was significantly more resistant to *N*-ethylmaleimide (Fig. 8*B*). The inhibition by low levels of salt is another characteristic of calf thymus pol δ (Fig. 8*C*). Recombinant p125 differed from the calf thymus enzyme in that it was less sensitive to inhibition. The Sf9 DNA polymerase activity was not inhibited but slightly stimulated at 100 mM KCl and was only inhibited at much higher salt concentrations (Fig. 8*C*). The heat inactivation of the three polymerases was also examined. The enzyme was heated to 45 °C and assayed for polymerase activity at the indicated times. DNA polymerase δ from calf thymus and the p125 subunit displayed a similar behavior when heat-treated and were much less sensitive to

TABLE I
Purification of recombinant DNA pol δ p125
Assays was performed using poly(dA) · oligo(dT) template.

Purification step	Protein	Activity	Specific activity	Recovery
	mg	*units*	*units/mg*	%
Cell extract	800	6,272	7.8	100
Phosphocellulose	26	936	36	15
Mono Q HR 5/5	2.2	616	280	20
ssDNA cellulose[a]	0.11	132	1,200	2

ssDNA, single-stranded DNA.

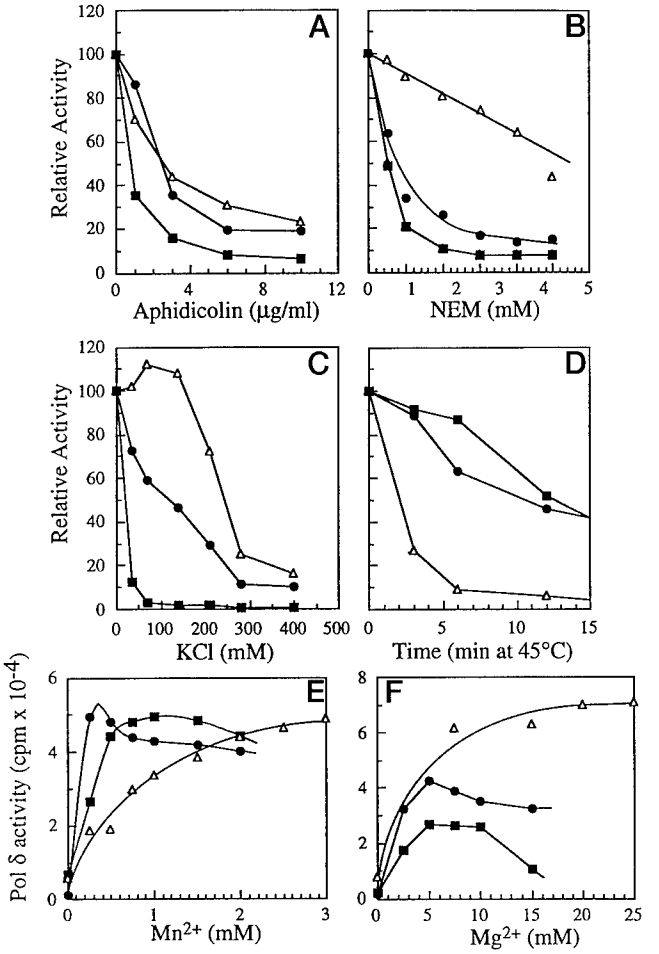

FIG. 8. **Characterization of recombinant pol δ: comparison with native calf thymus pol δ and endogenous DNA polymerases in baculovirus-infected Sf9 cells.** Effects of different compounds and conditions were assayed using poly(dA)·oligo(dT) as a template. Assay conditions were as described under "Experimental Procedures" for the DNA polymerase activities of recombinant pol δ (*closed circles*), native calf thymus pol δ (*closed squares*), and endogenous DNA polymerase from wild type baculovirus overexpressed in Sf9 cells (*open triangles*). PCNA was added in the assays for calf thymus pol δ. The endogenous DNA polymerase from wild type baculovirus overexpressed in Sf9 cells was the material obtained after phosphocellulose chromatography as in Fig. 4, *bottom panel*. *Panel A*, effect of aphidicolin; *panel B*, effect of *N*-ethylmaleimide; *panel C*, effect of KCl; *panel D*, effect of heat treatment at 45 °C for varying amounts of time; *panels E* and *F*, effects of Mn^{2+} and Mg^{2+}, respectively, on the DNA polymerase activity of recombinant pol δ.

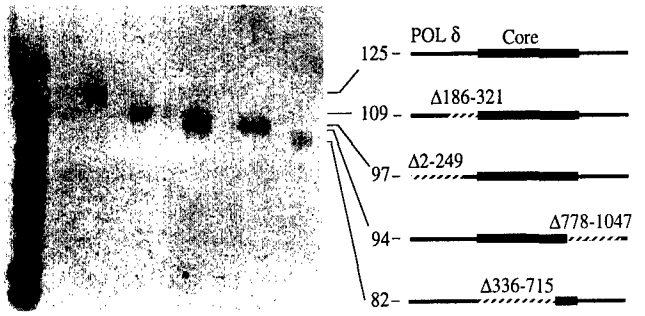

FIG. 9. **Expression of deletion mutants of pol δ p125.** Deletion mutants were constructed as described in Ref. 13. These mutants were purified to homogeneity by phosphocellulose, Mono Q, and single-stranded DNA-cellulose chromatography. The protein staining of the purified mutants after SDS-PAGE are shown. The map of the deletions is shown on the *right*.

heat than the Sf9 polymerase (Fig. 8*D*).

Recombinant pol δ was stimulated by Mn^{2+} in a manner similar to that already known for calf thymus pol δ. Optimal activation was observed between 0.3 and 0.5 mM Mn^{2+}, whereas optimal activity of the Sf9 polymerase was obtained at about 3 mM Mn^{2+} (Fig. 8*E*). Maximal activation of both calf thymus and recombinant pol δ by Mg^{2+} was reached at about 5 mM, whereas the Sf9 polymerase activity was stimulated maximally at 20 mM Mg^{2+} (Fig. 8*F*). These experiments showed that the properties of the recombinant p125 subunit were quite consistent with those of the calf thymus native enzyme.

Deletion Mutagenesis of p125—Extensive compilation and alignment of DNA polymerase sequences from a broad phylogenetic spectrum, *i.e.* from both prokaryotes and eukaryotes, have shown that these fall into two major protein families (16, 17). DNA pol δ belongs to the α-like or B family of DNA polymerases (16). A distinguishing feature of this family is the presence of a conserved core region containing six distinct conserved regions, I–VI, which are thought to contain the catalytic domain for polymerase activity. Unlike pol α, the NH_2-terminal regions of pol δ possess several regions (N1–N5) that are conserved in the Epstein-Barr virus and herpesvirus DNA polymerases (5).

Deletion mutants of the full-length human pol δ (1,107 residues) were constructed. These were p97, in which the N1 and N2 regions of the NH_2 terminus (2–249) were deleted; p109, in which N3, N4, and part of the N5 region including the *Exo*I domain (186–321) were deleted; p82, in which regions IV, A, B, II, VI, and III (336–715) were deleted; and p94, in which regions C, V, CT-1, CT-2, CT-3, and ZnF1 (778–1,047) were deleted (7). These were purified to near homogeneity by phosphocellulose, Mono Q, and single-stranded cellulose chromatography as described above. SDS-PAGE of the mutants (Fig. 9) showed that these had the expected molecular weights. Assays for enzyme activity showed that only p109 (Δ186–321) and p97 (Δ2–249) retained DNA polymerase activity. The p82 and p94 mutants had negligible activities (Table II). This is expected as most of the core region involved in deoxynucleotide interaction was deleted in p82, whereas most of the COOH-terminal domain responsible for DNA interaction was deleted in p94 (Fig. 9).

Evidence for the Phosphorylation of Pol δ by Cyclin-dependent Protein Kinases—Sf9 cells were coinfected with recombinant viruses harboring pol δ and different pairs of recombinant baculoviruses harboring cdk-cyclins. The cdk-cyclin pairs were cdk2-cyclin A, cdk2-cyclin E, cdk4-cyclin D1, cdk4-cyclin D2, cdk4-cyclin D3, cdc2-cyclin A, and cdc2-cyclin B1. After 48 h of infection, the cells were labeled with $^{32}P_i$ for 2 h at 37 °C in low phosphate medium, sonicated, and analyzed by immunoprecipitation using a mixture of pol δ monoclonal antibodies followed by SDS-PAGE and autoradiography as described previously (13). The results (Fig. 10) showed that pol δ was hyperphosphorylated when it was coexpressed with the G_1 phase-specific cdk-cyclins, cdk4-cyclin D3 or cdk2-cyclin E. The relative intensity of phosphorylation when pol δ was coexpressed with these cdk-cyclins was about 10-fold greater than when pol δ was expressed on its own. The relative phosphorylation of pol δ after coinfection with the S or G_2/M-specific cdc2-cyclins (cdc2-cyclin A or cdc2-cyclin B1) was about 20% of that of the G_1/S-specific cdk-cyclins. Cdk2-cyclin A and cdk4-cyclin D2 gave phosphorylation intensities that were similar to the control values obtained when pol δ was expressed alone. The relative intensity of cdk4-cyclin D1 coinfected with pol δ was lower than that of pol δ alone. Our results indicate that pol δ is phosphorylated by cdk4-cyclin D3 and cdk2-cyclin E and is a likely substrate of these G_1/S-specific cdk-cyclins.

TABLE II
Relative specific activities of recombinant p125 and its deletion mutants

Enzymes were purified to near homogeneity as described under "Experimental Procedures" and assayed for DNA polymerase activity using poly(dA) · oligo(dT) as template.

Enzyme	Protein[a]	Specific activity	Relative specific activity
	mg/ml	*units/mg*	
p125	0.0168	1,270	100
Δ186–321	0.0178	1,290	102
Δ2 –249	0.0150	896	71
Δ336–715	0.0195	<0.01	<0.01
Δ778–1047	0.0187	<0.01	<0.01

[a] Concentration of protein in the final preparation was assayed using Coomassie Blue.

Activity of Phosphorylated and Unphosphorylated Forms of Pol δ—The effects of coexpression of p125 with cdk2-cyclin E, cdk2-cyclin A, and cdk4-cyclin D3 on the activity of pol δ were assessed by examination of the activities in the lysates after gel filtration on an HPLC column (Table III). There were no striking effects on the specific activities of the pol δ catalytic subunit assayed using poly(dA)·oligo(dT) as a template (Table III). Immunoblots for the cdk-cyclins in the fractions confirmed that these were also present in the fractions.

Coimmunoprecipitation of Cdk2 and Pol δ—It was found that pol δ could be coimmunoprecipitated with cdk2 from Sf9 cell extracts when they were coexpressed in experiments in which the extracts were immunoprecipitated with antibody against cdk2 and immunoblotted with antibody against pol δ (not shown). The interaction of pol δ with cdk2 was investigated further by examination of the coimmunoprecipitation of deletion mutants of pol δ with cdk2. The results (Fig. 11) showed that all of the deletion mutants tested were coimmunoprecipitated with the exception of the mutant in which the NH_2 terminus (residues 2–249) were deleted. These results demonstrate that there is likely a direct interaction between cdk2 and pol δ, although the possibility that this interaction is mediated by a third protein cannot be discounted.

Coimmunoprecipitation of Pol δ with Members of the Cdk-Cyclins—The coimmunoprecipitation of pol δ with cdk2 could also be observed in cultured Molt 4 cell extracts when cell extracts were immunoprecipitated with pol δ antibody and Western blotted with antibody to cdk2 (Fig. 12, *first lane*). The reciprocal experiment using cdk2 as the precipitating antibody followed by immunoblotting with pol δ antibody also showed that cdk2 was coimmunoprecipitated with pol δ (Fig. 12, *last lane*). When cyclin E was used as the precipitating antibody, the coimmunoprecipitation of pol δ was observed. The coimmunoprecipitation of cdk2 and cdk5 by PCNA antibody was also observed under the same experimental conditions (Fig. 12).

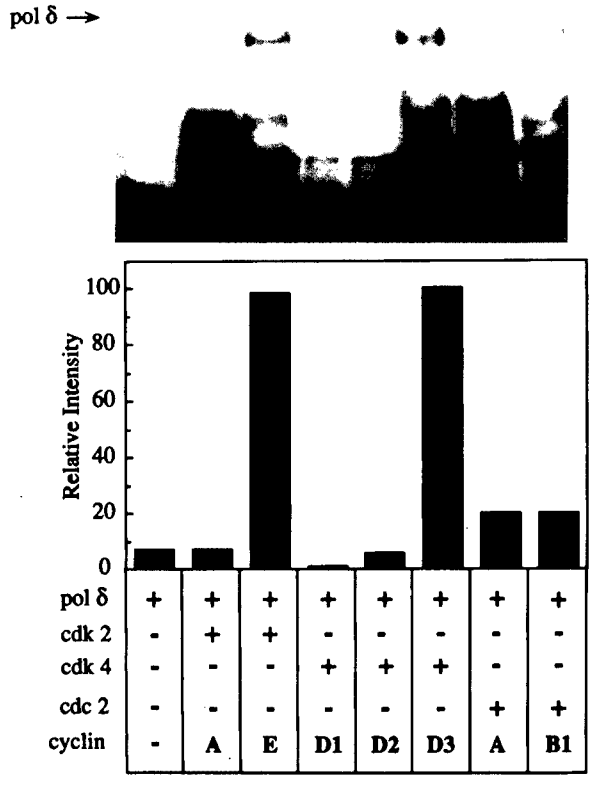

FIG. 10. ***In vivo* phosphorylation of recombinant pol δ in Sf9 insect cells.** The indicated cdk-cyclins and pol δ were coexpressed in Sf9 cells by coinfection as described under "Experimental Procedures." The cells were labeled metabolically with $^{32}P_i$, and the cell lysates were immunoprecipitated with 20 μg of pol δ monoclonal antibody and 40 μl of protein A-Sepharose slurry. The immunoprecipitates were subjected to SDS-PAGE and then autoradiographed (*upper panel*). Relative intensities of the pol δ p125 polypeptide were determined by densitometry.

TABLE III
Specific activities of p125 coinfected with different combinations of cdk-cyclins

Lysates obtained from equal amounts of coinfected cells were precipitated with 50% ammonium sulfate. The precipitates were dissolved in TGEED buffer containing 150 mM KCl, and equal volumes (0.5 ml) of each were loaded onto a Superose 6 HPLC gel filtration column (see "Experimental Procedures"). The results show the protein concentration and pol δ activities of the peak fractions. The presence of the cdk-cyclins in the eluates was confirmed by immunoblot (not shown).

Cotransformant	Protein concentration	Specific activity
	mg/ml	*units/mg*
None	0.33	34
Cdk2	0.36	48
Cdk2-cyclin A	0.36	61
Cdk2-cyclin E	0.79	38
Cdk4-cyclin D3	0.3	34

These experiments show that pol δ interacts with cdk2 and a cyclin *in vivo* and point to the existence of macromolecular complexes between pol δ and the cdk-cyclins.

DISCUSSION

The studies reported here show that the catalytic subunit of DNA pol δ can be expressed in Sf9 cells in an active form and can be isolated by a conventional purification protocol or by an immunoaffinity chromatography procedure. Isolation of the recombinant protein was aided by the use of antibodies against pol δ which did not cross-react with the endogenous DNA polymerase in baculovirus-infected Sf9 cells. We took advantage of an immunoaffinity chromatography procedure to purify

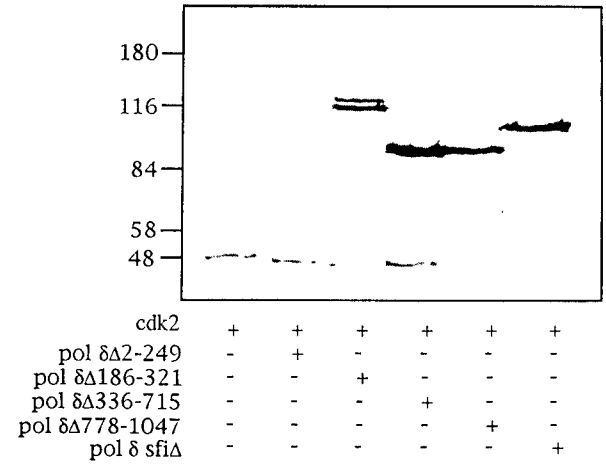

FIG. 11. **Analysis of the ability of the deletion mutants of pol δ to bind to cdk2.** Sf9 cells (about 10^7) were coinfected with pol δ deletion mutants and cdk2 recombinant baculoviruses as indicated. The levels of expression of these mutants were similar as determined by immunoblotting of the Sf9 cell lysates. About 10 mg of total protein from each cell lysate was used for immunoprecipitation with cdk2 polyclonal antibody and SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and immunoblotted with a mixture of NH_2- and COOH-terminal pol δ monoclonal antibodies.

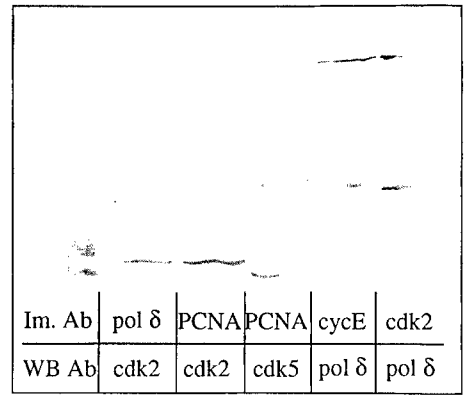

FIG. 12. **Coimmunoprecipitation of pol δ with members of the cdk-cyclin system.** Molt 4 cells were lysed by sonication. About 10 mg of total protein was immunoprecipitated with the first antibody (*Im. Ab*) plus protein A-Sepharose and then Western blotted with a second antibody (*WB Ab*). The *common band* in the *last three lanes* is an artifact (IgG heavy chain).

the recombinant pol δ in a facile manner and to ascertain that it was separated from any endogenous DNA polymerases. The properties of the overexpressed p125 catalytic subunit were compared with those of the native enzyme. Assays of the enzyme activity using poly(dA)·oligo(dT) as a template showed that the specific activities of the preparations were only about 1,200 units/mg (Table I) compared with about 25,000 units/mg protein for the calf thymus holoenzyme (14). This difference is likely the result of the lack of, or of a greatly attenuated response to PCNA by the free catalytic subunit. Other studies of pol δ preparations containing only the catalytic subunit have suggested that it is not PCNA-responsive (18, 19), whereas our previous studies of recombinant pol δ expressed in vaccinia virus have indicated a weak response (2–3-fold stimulation). The baculovirus-expressed pol δ shows little or no response to PCNA, whereas the response is restored by the presence of the p50 subunit (20–22). In other aspects, the enzymatic behavior of the recombinant p125 is very similar to that of the holoenzyme.

Studies of deletion mutants show that deletions (amino acids 2–249 or 186–321) in the NH_2 terminus retain polymerase

activity. Deletions in the core region (amino acids 336–715) and the deletion of regions C and V in the core as well as most of the COOH-terminal region including the zinc finger motifs (778–1047) had no assayable activity (Table II). This is consistent with numerous other studies that indicate that the core region of this family of polymerases is involved in the binding of the incoming dNTP substrate (23, 24) and contains the catalytic center for DNA polymerase activity. The retention of enzymatic activity by the NH_2-terminal deletion mutants is consistent with the existence of a domain structure in which the NH_2-terminal region does not function in catalysis. That this is likely is also consistent with the structure of T4 polymerase, which contains most of the conserved core but only part of the NH_2-terminal region that includes a motif required for the exonuclease activity (5).

The present studies provide the first evidence that the catalytic subunit of pol δ is itself a substrate for cyclin-dependent protein kinases and that this is specific for the G_1 cdk-cyclins because other cdk-cyclin combinations were less effective in phosphorylating pol δ when they were coexpressed in Sf9 insect cells. Although the *in vivo* kinase activity of cdk-cyclin overexpressed in Sf9 insect cells may not reflect actual cellular events in the mammalian cell cycle, the involvement of G_1 phase cdk-cyclins is consistent with our previous observations that pol δ is phosphorylated *in vivo* during the cell cycle and is maximal near the G_1/S transition (25). The primary structure of pol δ shows a number of potential phosphorylation sites for the cdks, including six sites possessing the (S/T)P motif: serines 207 and 788 and threonines 83, 150, 238, and 640 (25). It is well known that in mammalian cells the key regulators of the transition from G_1 to S phase of the cell cycle include the G_1 cyclins-three D type cyclins (D1, D2, D3) and cyclin E (26). Cyclin E expression is periodic, peaks at the G_1/S transition, and regulates S phase commitment together with its catalytic subunit cdk2. Unlike cyclin E, expression of D type cyclins is cell lineage-specific and highly mitogen-dependent, rising on growth factor stimulation and declining rapidly on growth factor withdrawal (27, 28). The current model for G_1 cdk-cyclin functions is that cyclin D binds directly to the tumor suppressor gene product pRb, targeting cdk4 to its substrate, and resulting in phosphorylation of pRb during middle to late G_1 phase. This reverses the growth-suppressive effects of pRb by releasing transcriptional factor E2F from its inhibitory constraint; the untethered E2F factor is then able to activate a series of genes required for DNA replication (26). The G_1 cdk-cyclins are also thought to phosphorylate other key substrates resident at the DNA replication origin to trigger the actual onset of DNA replication once cells pass the restriction point (29, 30). Pol δ is the central enzyme in eukaryotic DNA replication and is tethered to DNA by a direct interaction with the PCNA clamp, which converts pol δ from a distributive into a highly processive enzyme for DNA synthesis (31, 32). Thus, the finding that pol δ is a substrate for the G_1 cdk-cyclins is of significance as it provides a potential linkage for the cell cycle control of DNA synthesis. However, our studies do not reveal any major effects of phosphorylation on the activity of the p125 catalytic subunit, and only small increases (<2-fold) were observed after coexpression with cdk-cyclins (Table III). Pol α-primase has also been shown to be phosphorylated, and phosphorylation does not or only moderately changes its enzymatic properties (33–35). However, the ability of pol α-primase to initiate SV40 DNA replication *in vitro* was found to be inhibited markedly after phosphorylation by cyclin A-dependent kinases (36).

Examination of the interaction of cdk2 with the deletion mutants of pol δ showed that the tertiary structure of pol δ is not required for this interaction and that the binding region is located in the NH_2-terminal 249 residues of pol δ. The NH_2-terminus of yeast and mammalian pol δ harbors several highly conserved regions (N1–N5) that are also present in herpes and Epstein-Barr viral polymerases (5). These conserved regions are likely protein-protein interaction sites for pol δ (5). The binding site of pol δ for PCNA has been mapped to the N2 region (13). The data presented also provide the first evidence for complexes that involve pol δ and the cdk-cyclins. The targeting of the cdks to a substrate has some precedence since the G_1 cdk-cyclins are known to form complexes with pRb. The obvious question is whether this has any functional physiological significance in relation to the phosphorylation or regulation of pol δ. The present findings show that the interaction of pol δ with cdk2 and cdk4 needs to be investigated further, in addition to the issue of the cellular role of phosphorylation of pol δ by the cdk-cyclins.

There are many levels at which phosphorylation could affect pol δ function other than the simple modulation of enzyme activity in a simple assay. This is apparent because physiologically pol δ is part of a holoenzyme and part of an extended multiprotein complex. Current findings that p21, a potent inhibitor of G_1 cdks, and pol δ compete for the same sites in the interdomain connector loop of PCNA (37, 38) add even more complexity to these questions. Xiong *et al.* (39, 40) observed that PCNA is in a quaternary complex that includes cyclin D, cyclin-dependent kinases (cdk2, cdk4, cdk5), and p21. No phosphorylation of PCNA and p21 was detected, suggesting that neither of them is the primary substrate of phosphorylation. Thus, there are many possible permutations and speculations possible as to how regulatory systems could emerge from this melange of potential complexes. We have obtained preliminary evidence that pol δ is a substrate for the cyclin-dependent protein kinases. This was shown by the coexpression of baculovirus vectors for pol δ with several different cdk-cyclin combinations in Sf9 cells (Fig. 10) and coimmunoprecipitation Western blot studies in Molt 4 cells (Fig. 12). These results suggest that more than one cyclin might regulate pol δ, possibly triggering its phosphorylation at different sites or times of the cell cycle. Coimmunoprecipitation of pol δ deletion mutants with cdk2 also established the site of interaction (Fig. 11). Although the regulation of pol δ by protein phosphorylation has yet to be demonstrated firmly, this possibility provides a potential mechanism that might provide for the temporal regulation of DNA synthesis in concert with the cell cycle.

Although the present evidence indicates that the phosphorylation status of the catalytic subunit of DNA polymerase δ may have no significant effect on its activity, the question of whether phosphorylation has any physiological relevance in affecting or regulating the biological function of polymerase δ still needs to be answered. A role of phosphorylation or binding of the kinase in affecting the properties of the polymerase *in vivo* in modulating the function of pol δ in DNA replication or repair cannot be excluded. In this regard, note that significant difference was observed when replication protein A is phosphorylated in SV40 DNA replication (41–43) and nucleotide excision repair systems (42). Further studies are needed to answer the question of the regulatory consequences of phosphorylation of pol δ and for that matter other replication proteins. The putative kinase consensus sequences in pol δ also show that it could be a substrate for DNA-dependent protein kinase. The latter kinase phosphorylates serine or threonine residues that are followed or preceded by glutamine residues (S/T)-Q or Q-(S/T). It remains to be determined whether other kinases, *e.g.* DNA-dependent protein kinase, are also involved in the phosphorylation of the catalytic subunit of pol δ.

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Identification of DNA replication and cell cycle proteins that interact with PCNA

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ABSTRACT

The identity of DNA replication proteins and cell cycle regulatory proteins which can be found in complexes involving PCNA were investigated by the use of PCNA immobilized on Sepharose 4B. A column containing bovine serum albumin (BSA) bound to Sepharose was used as a control. Fetal calf thymus extracts were chromatographed on PCNA–Sepharose and BSA–Sepharose. The columns were washed and then eluted with 0.5 M KCl. The salt eluates were examined for the presence of both DNA replication proteins (Pol α, δ, ε, PCNA, RFC, RFA, DNA ligase I, NDH II, Topo I and Topo II) and cell cycle proteins (Cyclins A, B1, D1, D2, D3, E, CDK2, CDK4, CDK5 and p21) by western blotting with specific antibodies. The DNA replication proteins which bound to PCNA–Sepharose included DNA polymerase δ and ε, PCNA, the 37 and 40 kDa subunits of RFC, the 70 kDa subunit of RPA, NDH II and topoisomerase I. No evidence for the binding of DNA polymerase α, DNA ligase I or topoisomerase II was obtained. Of the cell cycle proteins investigated, CDK2, CDK4 and CDK5 were bound. This study presents strong evidence that PCNA is a component of protein complexes containing DNA replication, repair and cell cycle regulatory proteins.

INTRODUCTION

The discovery of a stimulating factor for DNA polymerase δ (1,2) that eventually led to its identification as proliferating cell nuclear antigen (PCNA) (3) stimulated major advances in our understanding of DNA synthesis at the replication fork. PCNA functions as a sliding clamp which endows pol δ with a high degree of processivity (4). Studies of *in vitro* SV40 DNA replication have now led to a fuller understanding of the protein machinery required for the formation of a functional mammalian DNA replication fork in which DNA polymerase δ (pol δ) and PCNA play a central role. The current model is one in which replication factor C (RFC, also known as activator-1), a complex of five subunits, first binds to the primer-template terminus and loads the PCNA onto the 3′ hydroxyl end of the primer strand of the DNA primer-template in an ATP-dependent process. Following the formation of a RFC/PCNA complex, pol δ is then recruited to assemble an elongation complex that catalyzes DNA synthesis in the presence of deoxynucleotide triphosphates (5–9). Replication protein A (RPA), a ssDNA binding protein, is involved in both initiation and elongation, as it stimulates pol δ activity in the presence of RFC and PCNA (6,10). A DNA helicase activity is essential to the replication machinery and serves mainly to unwind replication origins during the initiation phase of DNA replication and to separate parental DNA strands during the elongation phase. A helicase which is highly associated with pol δ has been isolated (11). Recently, six human helicases have been purified to near homogeneity (12). Furthermore, a nuclear DNA helicase II (NDH II) has also been purified (13). Like the large T antigen of SV40, it was found to unwind both DNA and RNA. Molecular cloning of NDH II revealed a high homology to human RNA helicase A (14). Pol α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, while the actual elongation of the primers is performed by pol δ in a process requiring polymerase 'switching' (15). A topoisomerase activity is also required, and studies using the SV40 system showed that either topoisomerase I or topoisomerase II is capable of removing positive supercoils ahead of the replication fork (16,17). The model of the protein assembly at the replication fork now resembles that of the well defined prokaryotic systems, requiring the presence of two pol δ molecules (18).

A major area of research which is currently emerging is the exploration of the biochemical and genetic mechanisms by which cell cycle regulation of DNA synthesis is achieved. There have been rapid advances in delineating the existence of cell cycle proteins: these include the cyclins A and B, a family of G1 cyclins (E, D cyclins) and a family of cyclin dependent kinases (CDKs) (19,20). There is now evidence for the cell cycle control of mammalian DNA replication by the cyclin–CDK system (21). A number of studies point to the existence of protein–protein interactions of DNA synthesis proteins with cell cycle dependent protein kinases or cyclins, as well as the phosphorylation of DNA synthesis proteins by CDKs. DNA polymerase α is phosphorylated in a cell cycle specific manner and is a substrate for $p34^{cdc2}$ (22,23). The RPA complex purified from HeLa cells or Manca cells is also phosphorylated in a cell cycle dependent manner by one or more members of cyclin/CDK2 family, and its phosphorylation has been shown to stimulate the initiation of SV40 DNA synthesis *in vitro* (24,25). Recent studies by Pan *et al.* (26) showed that both CDK2/cyclin A and DNA-dependent protein

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kinase phosphorylate the 34 kDa subunit of RPA. However, phosphorylated and unphosphorylated forms of RPA were equally active in SV40 DNA replication and nucleotide excision repair (26). Using immunoprecipitation and western blot experiments, Xiong *et al.* (27,28) showed combinatorial interactions of D type cyclins, cyclin-dependent kinases with PCNA and with p21. p21, also known as WAF1, CIP1 or Sdi1, is an inhibitor of the CDKs that control the initiation of the S phase of the cell cycle and DNA replication. The N-terminal region of p21 contains the CDK inhibitory domain whereas the C-terminal region contains a PCNA binding domain that leads to the inhibition of DNA synthesis (29).

In addition, both pol δ and PCNA have been shown to be required for DNA repair (30). Thus, PCNA, through its interactions with elements of both the DNA replication apparatus and the cell cycle regulatory system, has emerged as an important locus for protein–protein interactions that may provide communication between DNA replication, DNA repair and cell cycle control. Definition of the number and nature of these protein–protein interactions will therefore be important. In this study, immobilized recombinant PCNA is used as a means for the isolation of proteins that bind to PCNA.

MATERIALS AND METHODS

Immunoblotting

After electrophoresis in 5–15% gradient gels, proteins were transferred to nitrocellulose membranes. Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and provided visual confirmation of efficient transfer. The nitrocellulose blots were incubated with 3% BSA in phosphate-buffered saline as a blocking agent. The blot was then incubated with the primary monoclonal antibody at a final concentration of 5 μg/ml or with a polyclonal antibody at ~1:500 dilution for 12 h at 25°C. After washing, the blot was incubated with biotinylated sheep anti-mouse immunoglobulin, followed by streptavidin-biotinylated peroxidase preformed complex. When polyclonal antibodies were used, the second antibody was anti-rabbit IgG biotinylated species-specific whole antibody instead of anti-mouse IgG. Color development was performed by incubation with 4-chloro-1-napthol and hydrogen peroxide and was terminated with sodium azide.

Antibodies used were as follows: polyclonal antibodies against the p145, p40, p37 and p38 subunits of RFC (Dr J.Hurwitz, Memorial Sloan Kettering Cancer Center, NY); polyclonal antibodies against the p70 and p11 subunits of RPA and monoclonal antibody against the p34 subunit of RPA (Dr S.H.Lee, St. Jude's Children's Hospital, Memphis, TN); polyclonal antibodies to human topoisomerase I and II (ToPoGen Inc.); PCNA monoclonal antibody mAB19F4 (American Biotech. Inc., Plantation, FL); p21 monoclonal antibody (Santa Cruz); monoclonal antibody against DNA polymerase ε (Dr J.E.Syvaoja, University of Oulu, Finland); monoclonal antibody against polymerase α (American Type Culture Collection); DNA ligase I polyclonal antibody (Dr A.Tomkinson, University of Texas Health Science Center at San Antonio); rabbit antiserum to mouse cyclins D1, D2 and D3 (Dr C.J.Sherr, St. Jude's Children's Hospital, Memphis, TN); monoclonal antibodies to cyclins A, B1 and E and to both CDK2 and CDK5 (Dr E.Lee, Massachusetts General Hospital, Boston); polyclonal antibody to CDK4 (Dr S.Hanks, Vanderbilt University, TN); monoclonal antibody to NDH II (Dr F.Grosse, Heinrich-Pette Institute for Experimental Virology and Immunology, Germany).

Preparation of PCNA and BSA affinity columns

Recombinant PCNA was overexpressed in *E.coli* and purified to homogeneity as previously described (31). Activated CH–Sepharose, which allows coupling to a six carbon spacer arm, was obtained from Pharmacia LKB Biotech. Purified recombinant PCNA (20 mg in 50 ml) was dialyzed against 2 l of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0 (four changes at 6–8 h intervals). Activated CH–Sepharose 4B (2 g) was suspended in 30 ml of cold 1 mM HCl. The gel was washed in a column with 400 ml of cold 1 mM HCl followed by 200 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0. The coupling reaction was performed by addition of PCNA (20 mg, 50 ml in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0) to the washed gel. The suspension was rotated end over end for 18 h at 4°C. The protein content of the supernatant was checked at intervals by absorbance at 280 nm to monitor the progress of the reaction. After an overnight reaction it was estimated that ~2 mg PCNA was bound/ml of gel. The suspension was centrifuged and the supernatant discarded. The gel was then suspended in 50 ml of 1 M ethanolamine, pH 9.0 for 18 h to block unreacted groups. The gel was washed with 200 ml of 1 M NaCl–0.1 M sodium acetate, pH 6.0 followed by 200 ml of 1 M NaCl–0.1 M Tris–HCl, pH 8.0 and 200 ml of 0.5 M NaCl–0.1 M Tris–HCl, pH 8.0. The gel was equilibrated with TGEED buffer (50 mM Tris–HCl, pH 7.8, 10% glycerol, 0.5 M EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). A control column in which bovine serum albumin (BSA) was substituted for PCNA was prepared by the same procedure. In this case ~8.4 mg of BSA were coupled to 5 ml of activated CH–Sepharose 4B. All operations were performed at 4°C.

Preparation of calf thymus extracts

Frozen fetal calf thymus tissue (10 g) was used to prepare 50 ml of tissue extract. The tissue was homogenized in a blender with 50 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.25 M sucrose, 10% glycerol, 10 mM KCl, 0.1 mg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mg/ml bacitracin, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride and 10 mM sodium bisulfite). The homogenate was centrifuged for 1 h at 15 000 *g*. The extract was then centrifuged at 100 000 *g* for 1 h. All operations were performed at 4°C.

Affinity chromatography

Affinity chromatography was performed by mixing 50 ml of calf thymus extract with the PCNA–Sepharose (5 ml) and rotating the suspension end over end for 2 h. The gel was then packed into a column and washed with 100 ml of 50 mM KCl in TGEED buffer and eluted with 0.5 M KCl in TGEED buffer. Fractions of 0.3 ml were collected. Control experiments in which immobilized BSA was used as the column support was performed in parallel.

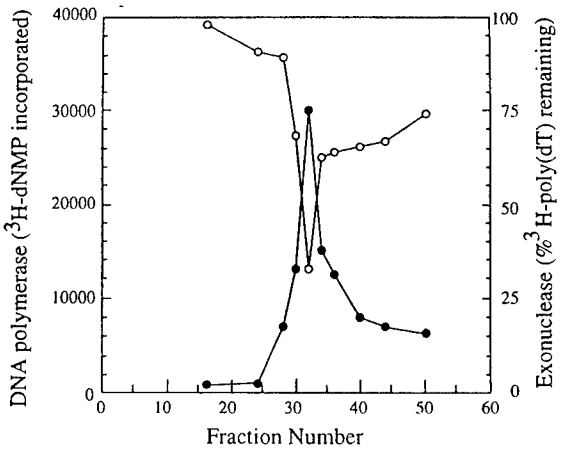

Figure 1. Affinity chromatography of calf thymus extract on PCNA–Sepharose. Crude calf thymus extract (50 ml) was rotated end over end with 5 ml of PCNA–Sepharose for 2 h. The gel was then packed onto a column, washed with 100 ml of 50 mM KCl TGEED buffer and stripped with 0.5 M KCl in TGEED (Materials and Methods). Fractions (0.3 ml) were collected and assayed for polymerase activity using poly dA/oligo dT as a template in the presence of PCNA (closed circles) and for exonuclease activity using [^{3}H]dT$_{50}$ (open circles) as previously described (1).

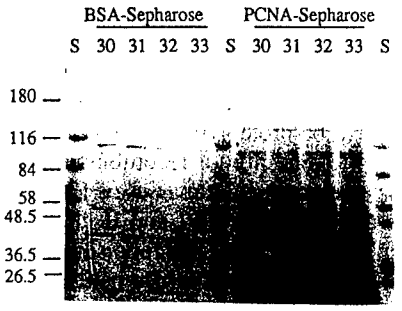

Figure 2. SDS–gel electrophoresis of proteins bound to PCNA and BSA affinity columns. Fractions 30–33 from the BSA-control column and the PCNA column were subjected to SDS–PAGE and stained for protein. From left to right are fractions 30–33 from the BSA control column, followed by fractions 30–33 from the PCNA column. The latter fractions correspond to the peak of pol δ activity. S: pre-stained protein standards (Sigma Chem. Co., α-2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose 6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48 kDa; lactate dehydrogenase, 36 kDa; triosephosphate isomerase, 26 kDa).

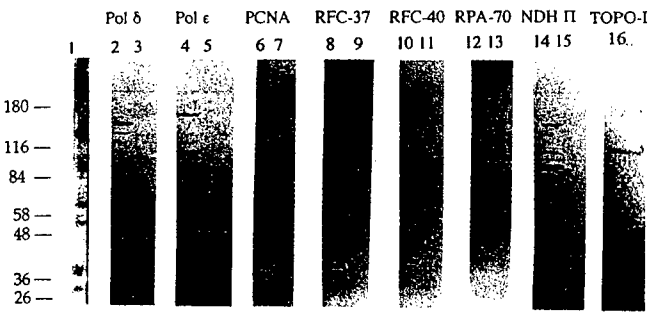

Figure 3. Immunoblots against DNA replication proteins bound to the PCNA affinity column. Lane 1: molecular weight markers with weights in kDa as indicated. Lanes 2, 4, 6, 8, 10, 12 ,14 and 16 each illustrate fraction 32 from the PCNA column western blotted against pol δ, pol ε, PCNA, RFC-37, RFC-40, RPA-70, NDH II and topoisomerase I antibodies, respectively. Lanes 3, 5, 7, 9, 11, 13 and 15 depict fraction 32 eluted from the BSA column and blotted against the same antibodies. Immunoblots were performed as described in Materials and Methods.

RESULTS

Affinity purification of DNA replication complex on PCNA–Sepharose

Calf thymus extracts were chromatographed on PCNA–Sepharose as described in Materials and Methods. The eluted fractions were assayed for DNA polymerase δ activity using poly dA/oligo dT as a template and for exonuclease activity using [^{3}H]dT$_{50}$. Preliminary experiments established that pol δ was bound to the column and was eluted at ~250 mM KCl when a KCl gradient was applied (not shown). A standard protocol was then used in which the bound material was eluted with 0.5 M KCl (Materials and Methods). No activity was detected in the flow through fractions, and both DNA polymerase and 3′→5′ exonuclease activities eluted together and were only detected in the eluate from the PCNA column (Fig. 1). (No activity was bound to a control BSA–Sepharose column when tested with a calf thymus extract.) The SDS–PAGE profile of polypeptides bound to PCNA–Sepharose and eluted with 0.5 M KCl is shown in Figure 2. A number of protein bands ranging from 18 to 210 kDa were present in the eluate from the PCNA–Sepharose column. The gels shown in Figure 2 were deliberately overloaded to show the presence of all bound polypeptides. Comparison with calf thymus extracts chromatographed on a control BSA column showed that there were several bands with two prominent polypeptides (110 and 43 kDa) that also adhered to the BSA column. Experiments were also performed in the presence of 1 mM ATP, since the interaction of PCNA with RFC is ATP dependent (6,7). However, the compositions of the polypeptides that were eluted were the same in the presence or absence of ATP (not shown).

The 0.5 M KCl eluate from the PCNA column was systematically tested for the presence of other replication proteins by western blotting. Representative blots are shown in Figure 3 from a number of individual experiments. The catalytic polypeptides of pol δ and ε were found to be present by western blot analysis using specific antibodies to pol δ (Fig. 3, lane 2) and pol ε (Fig. 3, lane 4). The pol δ antibody immunoblotted a band of 125 kDa, and the pol ε antibody detected a band of 145 kDa, in agreement with the previously reported molecular mass of pol ε isolated from calf thymus extracts (32,33). DNA polymerase α was not detected in the eluates by western blotting. PCNA itself was detected in the eluate as a 31 kDa band (Fig. 3, lane 6). This could be attributed either to stripping from the column, given that PCNA is trimeric, or due to an interaction of calf thymus PCNA subunits with immobilized PCNA. Antibodies against the individual 145, 40, 37 and 38 kDa subunits of RFC revealed positive results only for RFC-37 and RFC-40 (Fig. 3, lanes 8 and 10). Western blotting was also performed using antibodies to the 70, 34 and 11 kDa subunits of RPA. A positive blot was obtained for the 70 kDa subunit (Fig. 3, lane 12). These results indicate that both RFC and RPA are bound to the PCNA column.

The nuclear DNA helicase II (NDH II) enzyme was readily detected in the 0.5 M KCl eluate by immunoblotting as a 130 kDa band and three other bands of lower molecular weight ranging from 100 to 84 kDa (Fig. 3, lane 14). The lower molecular weight bands are likely to be proteolytic products. It has been reported that limited tryptic digestion of recombinant NDH II produced active helicases with molecular masses of 130 and 100 kDa (14). The presence of topoisomerase I and II, and ligase I was also tested for by immunoblotting. Only topoisomerase I was detected in the 0.5 M KCl eluate as a 100 kDa band (Table 1 and Fig. 3, lane 16). Examination of the fractions eluted from the BSA column by western blot yielded negative results for all of the above.

It is shown in this report that, in addition to pol δ, pol ε, RFC, RPA, PCNA, nuclear DNA helicase II (NDH II) and topoisomerase I are also present in the 0.5 M salt eluate from the PCNA column (Table 1). This collective elution of various constituents of the DNA replication machinery provides direct evidence for strong interactions between these proteins that directly or indirectly involve PCNA.

Table 1. Proteins which bind to immobilized PCNA as determined by western blotting of column eluates

Protein	PCNA column	BSA column	Protein	PCNA column	BSA column
Pol α	–	–	CDK2	+	–
Pol δ	+	–	CDK4	+	–
Pol ε	+	–	CDK5	+	–
PCNA	+	–	Cyclin A	–	–
RFC-37	+	–	Cyclin B1	–	–
RFC-38	–	–	Cyclin D1	–	–
RFC-40	+	–	Cyclin D2	–	–
RFC-145	–	–	Cyclin D3	–	–
RPA-11	–	–	Cyclin E	–	–
RPA-34	–	–	p21	–	–
RPA-70	+	–			
Ligase I	–	–			
NDH II	+	–			
Topo I	+	–			
Topo II	–	–			

Presence or absence in column eluates as determined by western blotting is shown as + or – respectively.

Binding of cell cycle regulatory proteins to PCNA–Sepharose

The PCNA–Sepharose column fractions containing peak pol δ activity were also tested for the presence of proteins involved in cell cycle regulation. Some principal components were detected through a series of western blots. Positive blots were obtained for cyclin dependent kinase 2 (CDK2), CDK4 and CDK5 (Fig. 4). Neither cyclins nor p21 were detected in the eluates (Table 1).

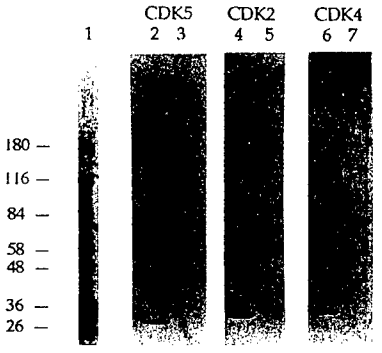

Figure 4. Immunoblots of cell cycle regulatory proteins eluting from the PCNA column. Lane 1: prestained protein markers with weights in kDa as shown. Lanes 2, 4 and 6: cell cycle regulatory proteins, CDK5 (31 kDa), CDK2 (33 kDa) and CDK4 (34 kDa), were bound by and eluted from the PCNA column. Shown also are the corresponding western blots of fraction 32 (lanes 3, 5 and 7) from the control BSA column where no CDK proteins were detected.

DISCUSSION

Recombinant PCNA was immobilized on Sepharose 4B and was systematically used to investigate the binding of replication and cell cycle proteins from fetal calf thymus extracts by affinity chromatography. As expected, tests for the binding of pol δ both by activity and by western blotting confirmed that it was bound. In addition, examination of the protein bands present in the peak of the bound fractions showed the presence of multiple polypeptide components. Some of these may represent adventitious binding although it may be noted that only a few bands with two prominent polypeptides of 110 and 43 kDa were observed in the eluates from the same fractions in the BSA control column. In addition to pol δ, several other replication proteins were bound to the affinity column. Specifically, the binding of PCNA, pol ε, RFC, RPA, NDH II and topoisomerase I was detected. The binding of pol δ and RFC was anticipated, since it is known that these proteins interact with PCNA (6). The collective elution of PCNA, pol δ, RFC and RPA was striking, in view of the fact that these are all components of the proposed replication complex involved in leading and lagging strand DNA synthesis (16,34).

The interaction of PCNA with pol ε is still controversial. Lee *et al.* (7) reported that RPA, RFC and PCNA could overcome the salt inhibition of DNA polymerase ε. Chui and Linn (35) observed strong inhibition of DNA polymerase ε by salt and found that this inhibition could not be completely overcome by RFC, RPA and PCNA which had little, if any, effect on the processivity of DNA polymerase ε. A major significance of these present findings is that they reveal a definite interaction, either direct or indirect, between pol ε and PCNA, thus linking pol ε to the replication fork. Navas *et al.* (36) have identified the DUN2 gene of *Saccharomyces cerevisiae* as DNA polymerase ε (Pol2). Mutations in the DUN2 gene displayed properties that suggest that pol ε has a role as a sensor of replication blocks and some forms of DNA damage, thus linking the DNA replication machinery to the S phase checkpoint (36). However, in *Schizosaccharomyces pombe* it was demonstrated that *cdc* 20^+ encodes the catalytic subunit of pol ε and the gene product is required for chromosomal replication but not for the S phase checkpoint (G.D'Urso, personal communication). A pol ε holoenzyme

consisting of pol ε, PCNA, RPA and RFC may function on the lagging strand of the replication fork (37). This could provide a mechanism for proofreading in the lagging strand because pol ε, unlike pol α and similar to pol δ, has a 3′→5′ exonuclease activity (38,39). Zlotkin *et al.* (40), using UV crosslinking of nascent cellular DNA and immunoprecipitation, showed that DNA polymerase ε is essential in cellular nuclear DNA replication. Studies of *S.pombe cdc* 20^+ mutants showed that pol ε plays an important role in the elongation of nascent DNA chains, suggesting that pol ε participates in the switch from primer extension by pol α primase to leading strand synthesis (G.D'Urso, personal communication).

The presence of topoisomerase I in the eluates from PCNA–Sepharose is interesting, as it functions to relieve positive superhelicity during replication (41). The positive immunoblots for nuclear DNA helicase II (NDH II) (Fig. 3) in the peak eluates reveals the possibility of a complex involving the helicase enzyme. This is interesting in view of the fact that there may be differences between viral and host chromosomal DNA replication, so that there may be limitations of the *in vitro* SV40 replication system as a model system. Recently, a human nuclear protein that interacts with the constitutive transport element (CTE) of simian retrovirus was identified as RNA helicase A (42). The latter has a high degree of similarity to NDH II which also has RNA helicase activity (14). RNA helicase A was found to be concentrated in the nucleus in normal cells (42). It was also identified as an inherent shuttling protein that interacts with CTE *in vitro* and associates with CTE in its trafficking from the nucleus to the cytoplasm *in vivo* (42). Whether the presence of NDH II is physiologically relevant in the DNA replication complex purified from the PCNA–Sepharose affinity column is still an open question.

These findings are in concert with, and support other studies which have led to the partial purification of macromolecular complexes using conventional protein purification methods (43–45). Immobilized T4 bacteriophage gene 32 protein has been successfully used to characterize and isolate the interacting components of the T4 replication complex (46). The existence of a physical assembly of a mammalian replication complex, the 'replisome' has been inferred from studies of the prokaryotic system, and by consideration of the requirement for processive and uninterrupted DNA synthesis during replication. Evidence for the existence of such complexes is still fragmentary, and is based on the isolation of partially purified protein fractions by conventional methods that contain a number of replication proteins that can functionally replicate viral DNA (18,34). The advantage of an affinity chromatography approach over conventional methods is that it is rapid and is based on protein–protein interactions. This greatly lessens concerns that these complexes may be artifacts of the isolation methods. The current studies using affinity chromatography demonstrates the existence of a system of protein–protein interactions involving the replication proteins that could provide the molecular basis for the formation of a replication complex.

Since PCNA has been reported to bind to the cyclins (27,28), the binding of the cyclins and associated cyclin dependent kinases to the PCNA–Sepharose was also tested. Blots for the cyclin dependent kinases were positive in the case of CDK2, CDK4 and CDK5, while tests for associated cyclins were negative. This is surprising, since both CDKs and cyclins have been reported to associate in quaternary complexes with PCNA and p21 (27,28). It may be that tissue levels of the cell cycle proteins in calf thymus were too low for detection (28); also, expression of p21 occurs as a result of DNA damage (47). In general, a failure to observe any given protein in our experiments does not preclude its involvement in a replication complex, since the experiments are dependent on the sensitivity of the antibodies, the strength of the association and the stability of the given protein–protein interaction during purification. Recent studies have described several intermolecular interactions between cell cycle proteins and the replication proteins that may be of mechanistic significance in the cell cycle regulation of DNA replication. These include the demonstration that cyclin A or cyclin E–CDK complexes can trigger initiation of DNA synthesis (48), and that cyclin A is required for *in vitro* DNA replication (49). The phosphorylation of replication proteins by cyclin/CDKs has been demonstrated in the case of HSSB-p34 (50). In the latter case, cyclinA/CDK2 but not cyclinE/cdk2 was shown to phosphorylate HSSB. This critical observation indicates that targeting of the CDK2 to HSSB is necessary for phosphorylation to occur. Pol δ was reported to be phosphorylated *in vivo* (51). Recently, pol δ was found to be phosphorylated by cyclin/CDKs (Zeng and Lee, unpublished observations).

These studies which show the binding of both replication and cell cycle proteins to PCNA provides additional support for a central role of PCNA in the linkage of the processes of DNA replication and cell cycle regulation via protein–protein interactions. In addition, these findings demonstrate the existence of protein–protein interactions between DNA replication proteins and cell cycle regulatory proteins. This interaction of the cyclin–CDK cell cycle regulatory proteins with polymerases and elements of the DNA replication system could be important in understanding the cell cycle control of DNA replication. The findings that PCNA exhibits interaction with multiple protein partners suggest that it may have an important role in the formation of macromolecular complexes involved in DNA replication and its cell cycle control. For this reason, immobilized PCNA may be a particularly useful tool for the isolation of these complexes, a view that is supported by the studies reported. A molecular basis for the multiple protein partners with which PCNA interacts is now emerging, in the form of the recent identification of a short peptide consensus sequence which is found in several PCNA binding proteins including p21 (52), Fen1 (53) and cdc27 (54). There remain major questions as to the number and nature of the interacting protein partners of PCNA, and the mechanisms of how these interactions provide the necessary functional and regulatory outcomes in DNA replication and repair.

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Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA Polymerase δ*

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The formation of a complex between DNA polymerase δ (pol δ) and its sliding clamp, proliferating cell nuclear antigen (PCNA), is responsible for the maintenance of processive DNA synthesis at the leading strand of the replication fork. In this study, the ability of the p125 catalytic subunit of DNA polymerase δ to engage in protein-protein interactions with PCNA was established by biochemical and genetic methods. p125 and PCNA were shown to co-immunoprecipitate from either calf thymus or HeLa extracts, or when they were ectopically co-expressed in Cos 7 cells. Because pol δ is a multimeric protein, this interaction could be indirect. Thus, rigorous evidence was sought for a direct interaction of the p125 catalytic subunit and PCNA. To do this, the ability of recombinant p125 to interact with PCNA was established by biochemical means. p125 co-expressed with PCNA in Sf9 cells was shown to form a physical complex that can be detected on gel filtration and that can be cross-linked with the bifunctional cross-linking agent Sulfo-EGS (ethylene glycol *bis* (sulfosuccinimidylsuccinate)). An interaction between p125 and PCNA could also be demonstrated in the yeast two hybrid system. Overlay experiments using biotinylated PCNA showed that the free p125 subunit interacts with PCNA. The PCNA overlay blotting method was also used to demonstrate the binding of synthetic peptides corresponding to the N2 region of pol δ and provides evidence for a site on pol δ that is involved in the protein-protein interactions between PCNA and pol δ. This region contains a sequence that is a potential member of the PCNA binding motif found in other PCNA-binding proteins. These studies provide an unequivocal demonstration that the p125 subunit of pol δ interacts with PCNA.

Proliferating cell nuclear antigen (PCNA)[1] was originally discovered as an antigen in autoimmune sera from patients with systemic lupus erythematosus and was reported to be found only in actively proliferating cells (1). It was later shown to be a factor that enhanced the processivity of DNA polymerase δ (pol δ) and to have key roles in both DNA replication and repair (2, 3). There have been striking recent advances in our understanding of the structure and functions of PCNA (4). Purification and expression of human recombinant PCNA and its physiochemical characterization established that it was a trimeric protein (5). The crystal structures of both yeast and human PCNA have been determined (6, 7). Like the T4 gene 45 protein and the β subunit of *Escherichia coli* DNA polymerase III holoenzyme, PCNA functions as a sliding DNA clamp that forms a closed ring around duplex DNA (8). The binding of pol δ to PCNA provides an elegant micromechanical solution to the biological need to maintain an extraordinarily high level of processivity during the synthesis of chromosomal DNA (8–10). Recently, it has also been found that PCNA has a number of protein partners with which it interacts (4, 9, 11). Pol δ has been shown to be involved not only in DNA replication but also in DNA repair and can be regulated by cell cycle proteins (2, 12, 13). Thus, an important area of interest is the protein-protein interaction sites of PCNA, because it may be the nexus for multiple protein-protein interactions involved in replication, repair, recombination, and cell cycle regulation.

The pol δ core enzyme consists of two subunits, p125 and p50 (14). Previous work from this laboratory has implicated the p125 subunit in an interaction with PCNA (10). A synthetic peptide conforming to the N2 region (residues 129–149) was found to inhibit PCNA stimulation of pol δ isolated from calf thymus (10). p125 and PCNA co-expressed in Sf9 cells could be co-immunoprecipitated with an antibody to PCNA, showing that the catalytic subunit of DNA polymerase δ interacted with PCNA (10). However, the recombinant p125 catalytic subunit can only be stimulated by PCNA at most 2–3-fold, and the presence of the p50 subunit is required to restore a significant level of PCNA stimulation of the p125 subunit (15–17).

Two recent studies of yeast pol δ reported contrary results. No evidence for a direct interaction between the *Schizosaccharomyces pombe* pol δ p125 subunit and PCNA could be found, either by co-immunoprecipitation experiments after their co-expression in insect cells or by a yeast two hybrid assay (18). It was concluded that no direct interaction occurs between *S. pombe* p125 and PCNA. In *Saccharomyces cerevisiae*, similar results were obtained using a PCNA overlay assay. In the latter studies, the interaction of *S. cerevisiae* p125 and p58, as well as the recently identified third subunit (p55), was studied by a PCNA overlay method. It was shown that only the third subunit (p55) of pol δ interacted with PCNA (19), indicating that the interaction of pol δ with PCNA involved the third subunit, whereas no evidence could be obtained for an interaction of PCNA with either the small second subunit p58 or the catalytic subunit.

These findings are in conflict with our previous studies of the p125-PCNA interaction, and they suggest that the binding of PCNA in yeast and human depend on different subunit-PCNA interactions. Alternatively, it is also possible that the interaction of PCNA with pol δ involves multiple interactions with pol

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; pol δ, polymerase δ; EGS, ethylene glycol-*bis*(sulfosuccinimidylsuccinate); PAGE, polyacrylamide gel electrophoresis.

δ subunits. For this reason, we have undertaken a rigorous examination of the protein-protein interactions of the p125 subunit of pol δ and PCNA using different biochemical methods. Our studies leave little doubt of the ability of the p125 subunit to interact with PCNA.

EXPERIMENTAL PROCEDURES

Ectopic Expression of p125 and PCNA in COS-7 Cells—COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum. These cells were transfected with c-Myc-His-PCNA and pCMV-p125 using the calcium phosphate method. For each 9-cm Petri dish, the transfected DNA consisted of 5 μg of each expression vector and 10 μg of Bluescript SK DNA. The cells were washed with phosphate-buffered saline and scraped in 1 ml of radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). After 15 min on ice, the lysed cells were spun at 5000 rpm for 15 min at 4 °C. Immunoprecipitations were performed using anti-Myc antibody (Roche Molecular Biochemicals) in 1 ml of extract. The immunoprecipitates were separated on a 5–15% gradient SDS gel and immunoblotted with anti-His antibody (Invitrogen) or pol δ monoclonal antibody. The blot was then detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Gel Filtration of Sf9 Lysates Expressing Recombinant p125 and PCNA—Sf9 cells were grown in 75-cm^2 flasks to 75% confluence. The cells were dislodged from 25 flasks by pipetting 5 ml of fresh medium into the flasks. Cell pellets were collected by centrifugation at 3000 × *g* for 10 min. Recombinant baculoviruses containing the human p125 coding sequence, its deletion mutants, or PCNA were as described previously (13). Sf9 cells were infected with either the p125 or the deleted p125 and PCNA at a multiplicity of infection of 5 for each recombinant baculovirus. The cells were incubated in the viral inoculum for 2 h with gentle rotation every 15 min and then centrifuged at 3000 × *g* for another 10 min. The inoculum was removed, and cell pellets were suspended in 150 ml of fresh medium divided into 2 flasks and incubated for another 60 h.

The cell lysates from a total of 2 × 10^8 cells were collected and processed as described previously (13). One mg of protein in a total volume of 0.5 ml was chromatographed on a Sephacryl S-300 column (1.5 × 100 cm). Fractions of 1.5 ml were collected. The elution of pol δ activity was monitored by its activity on poly(dA)/oligo(dT) as described previously (10)

Cross-linking of p125 and PCNA Expressed in Sf9 Cells—The peak fraction of the PCNA-p125 complex from the S-300 chromatography was then subjected to cross-linking with Sulfo-EGS at the indicated concentrations for 15 min at room temperature as described previously (5). The cross-linked species were identified by Western blotting. Western blot was performed using 78F5 and 38B5 pol δ monoclonal antibodies (13). Prestained protein standards (Sigma) were used as molecular weight markers and also to provide for visual confirmation of efficient transfer. Nitrocellulose blots were blocked in 5% (w/v) nonfat dry milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST) for 1 h at room temperature. The blot was then incubated with monoclonal antibodies to pol δ for 12 h. at 25 °C. After three 10-min washes in TBST, the blot was incubated with anti-mouse IgG-horseradish peroxide conjugate diluted in TBST (1:10,000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

Overlay Blotting with Biotinylated PCNA—Recombinant PCNA was expressed in *E. coli* and purified to near homogeneity as described previously (5). PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (Amersham Pharmacia Biotech). The reaction mixtures contained 0.5 mg of PCNA, 0.5 mg/ml biotinamidocaproate *N*-hydroxysuccinamide ester in a total volume of 0.5 ml in 20 mM bicarbonate buffer, pH 8.6. After reaction for 60 min at room temperature, the PCNA was purified on a G25 Sephadex column (5-ml bed volume) equilibrated in phosphate buffered saline containing 1% bovine serum albumin. Samples containing 1–5 μg of protein were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Prestained protein standards (New England Biolabs) were used as molecular weight markers. The nitrocellulose membranes were blocked with 5% nonfat dry milk in TBST for 45 min at room temperature followed by three washes of TBST for 10 min each. The blots were then incubated with biotinylated PCNA (1 μg/μl) diluted in TBST (1:900) at 4 °C overnight. The blots were washed five times with TBST for 15 min followed by incubation with streptavidin-horseradish peroxidase conjugate diluted in TBST (1:5000) for 1 h at room temperature with constant rocking. The blots were then washed five times with TBST for 20 min each and developed using by chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

Dot Blot Analysis of Binding of PCNA to Synthetic Peptides—Peptides to p21 and the N2 region of pol δ p125 were synthesized by Bio-Synthesis Co. The peptides were dissolved in water at a concentration of 5 μg/μl. Five μl of each peptide were dot blotted onto nitrocellulose membranes. Bovine serum albumin, PCNA, p125 and purified calf thymus pol δ (25 μg) were dot blotted onto the same membranes. The nitrocellulose membranes were then blocked and blotted with biotinylated PCNA as described above.

Pairwise Yeast Two Hybrid Interactions—Plasmids expressing the GAL4 fusions with the coding sequences for p125, p50 and PCNA were constructed in the pAS2–1 and pACT2 vectors (CLONTECH Laboratories Inc.). Sequential transformations of Y190 were performed by the lithium acetate method with dimethyl sulfoxide at a final concentration of 10%. pAS2–1-p125 and pAS2-p50 were transformed into Y190 and plated onto Trp$^-$ plates. Y190 stains transformed with pAS2–1-p125 or pAS2–1-p50 were then transformed with pACT2–50 or pACT2-PCNA. After overnight recovery in Trp$^-$Leu$^-$ medium, the transformants were plated on Trp$^-$Leu$^-$ His$^-$/3-AT plates to select for histidine prototrophy. For the liquid assay, GAL4 and T antigen/p53 were transformed into Y190 as positive controls. pAS2–1-p50 and pAS2–1-PCNA in Y190 were used as negative controls. One ml of overnight yeast culture in liquid Trp$^-$Leu$^-$His$^-$/SD selection medium was prepared. Four ml of YPD medium (yeast culture medium containing 20 g/liter Difco peptone, 10 g/liter yeast extract, and 2% glucose) was added to the overnight culture and incubated for 5 h at room temperature with shaking (230–250 rpm). The A_{600} of the culture was recorded. One ml of cells was centrifuged at 1,000 × *g* for 30 s and washed once with 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgCl_2 \cdot 7H_2O$) followed by another centrifugation. The cell pellet was resuspended in 100 μl of Z buffer. The cells were disrupted by freeze-thaw (liquid nitrogen for 1 min, thawed at 30 °C for 1 min). Z buffer (0.7 ml) and β-mercaptoethanol at a final concentration of 40 mM was added. *O*-Nitrophenyl β-D-galactopyranoside (160 μl, 2.2 mM in Z buffer) was added, and the reaction was incubated at 30 °C for 3–90 min. (Positive strong interactions were incubated for 3 min and negative interactions for 90 min.) The reactions were terminated by addition of 0.4 ml of 1 M Na_2CO_3. Cell debris was removed by centrifugation at 10,000 × *g* for 2 min, and the A_{420} was recorded. The β-galactosidase activity was calculated. Arbitrary units of activity were calculated as: β-galactosidase units = $1000 \times A_{420}/(t \times V \times A_{600})$, where t = min of incubation; V = 0.1 ml.

RESULTS

Co-immunoprecipitation of p125 and PCNA from Crude Calf Thymus and HeLa Extracts and after Their Ectopic Expression in Cultured Cells—Previous studies have demonstrated that human PCNA could be co-immunoprecipitated with the p125 catalytic subunit of pol δ from Sf9 insect cell lysates, under conditions where both proteins were overexpressed as recombinant proteins (10). It can be argued that the interactions observed were a consequence of the supranormal concentrations of both proteins under these conditions of overexpression and may not reflect the behavior of the two proteins in a normal cellular context. Studies were therefore performed to establish whether PCNA and p125 could be co-immunoprecipitated from mammalian tissue or cell culture extracts. The results show that p125 and PCNA can be readily co-immunoprecipitated from crude calf thymus extracts that had been partially purified on phenyl agarose, as well as from HeLa cell lysates (Fig. 1, *A* and *B*). Because of the multisubunit nature of pol δ, these experiments do not show a direct interaction between p125 and PCNA, but they do confirm that an interaction between pol δ and PCNA is readily demonstrated in cell extracts.

Next, evidence for an interaction between p125 and PCNA when they are ectopically expressed in mammalian cultured cells was sought. Human PCNA was expressed in Cos 7 cells with dual tags (a hexahistidine tag and a c-Myc tag) together with p125 expressed in the pCMV vector (see under "Experimental Procedures"). Samples of the cell extracts were immu-

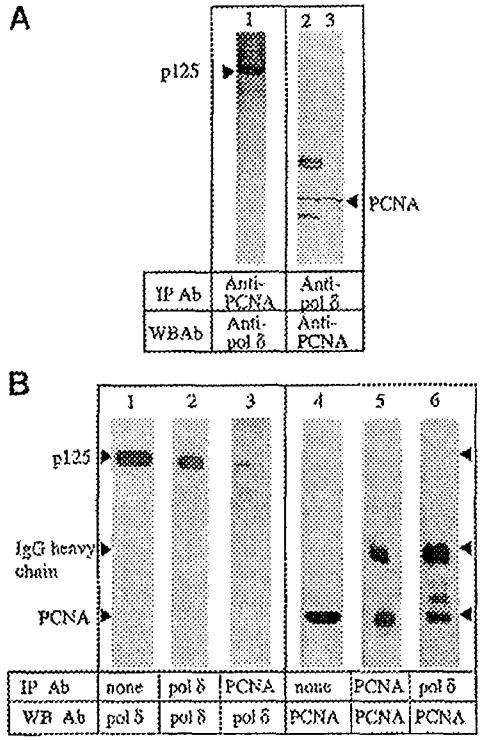


FIG. 1. **Co-immunoprecipitation of p125 and PCNA with monoclonal antibodies from calf thymus extracts or HeLa extracts.** *A, lane 1,* a calf thymus extract was passed through phenyl agarose, and 50 μl of the extract was immunoprecipitated (*IP*) with 20 μg of 74B1 PCNA monoclonal antibody and blotted with monoclonal antibody 78F5 against pol δ. *Lane 2,* a calf thymus extract was passed through phenyl agarose, and 50 μl of the extract was immunoprecipitated with 78F5 pol δ antibody and immunoblotted with 20 μg of 74B1 monoclonal antibody against PCNA. *Lane 3,* control containing purified PCNA, immunoblotted with the antibody against PCNA. Western blotting (*WB*) was performed using biotinylated sheep anti-mouse immunoglobulin as second antibody followed by incubation with streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide. *B,* HeLa cells were lysed in 1% Nonidet P-40 lysis buffer and protease inhibitors. The lysate was precleared with protein G agarose bead before incubation with antibodies (*Ab*). HeLa lysate (2 mg of protein) was incubated with 30 μg of monoclonal antibody for each immunoprecipitation. Immunobeads were suspended in 150 μl of lysis buffer and 150 μl of 2× SDS loading buffer. *Lanes 1* and *4,* 50 μg of untreated crude HeLa lysate. *Lanes 2* and *6,* 50 μl of immunoprecipitate from monoclonal 38B5 against pol δ. *Lanes 3* and *5,* 50 μl of immunoprecipitate from monoclonal antibody 74B1 against PCNA. Samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was divided into two halves. *Lanes 1–3* were immunoblotted with monoclonal antibody 78F5 against pol δ. *Lanes 4–6* were immunoblotted with monoclonal antibody 74B1 against PCNA. Western blotting was performed using horseradish peroxidase anti-mouse immunoglobulin as a second antibody followed by chemiluminescence detection.

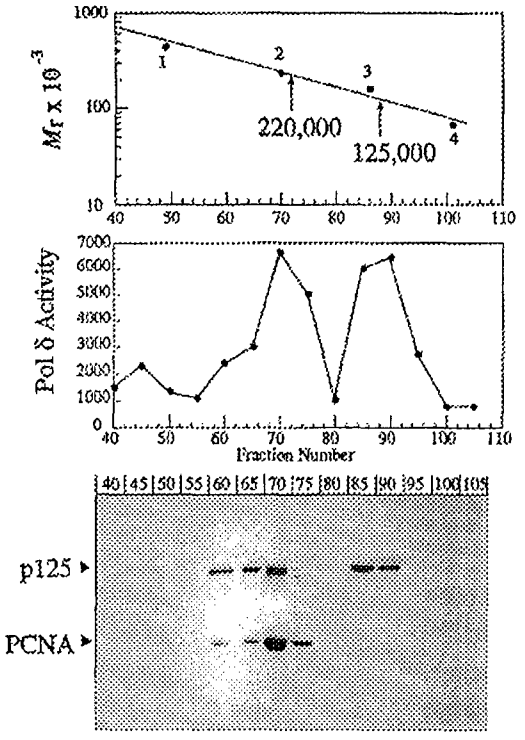


FIG. 2. **Gel filtration of Sf9 lysates expressing recombinant p125 and PCNA.** p125 and PCNA were co-expressed in Sf9 cells, and the cell lysates (0.5 ml, 1 mg of protein) were chromatographed on Sephacryl S-300 (see under "Experimental Procedures"). The elution of pol δ activity was monitored by activity assay (*center panel*) and for p125 and PCNA by Western blotting using a mixture of monoclonal antibodies to p125 and PCNA (*bottom panel*). The column was calibrated using ferritin (molecular weight 440,000), catalase (232,000), aldolase (158,000), and bovine serum albumin (67,000), shown as *1–4* in the *upper panel*. The approximate molecular weights of the two peaks of pol δ activity were 220,000 and 125,000, as shown by the *arrows* in the *upper panel*.

noprecipitated using a c-Myc antibody and were Western blotted with pol δ antibody. The results show that p125 is co-immunoprecipitated with c-Myc-His-tagged PCNA (not shown). Control experiments in which the immunoprecipitates were blotted with anti-His antibody confirmed that PCNA was present in the immunoprecipitate. These experiments demonstrate that pol δ p125 can be shown to co-immunoprecipitate with PCNA, either when endogenous pol δ is present or when p125 is ectopically co-expressed with PCNA.

Formation of a Physical Complex between p125 and PCNA—The experimental results in which p125 co-expressed with PCNA in either baculovirus (10) or in mammalian cells (Fig. 1) indicated that p125 is able to interact with PCNA and imply that this interaction is independent of the presence of p50 or other subunits of pol δ. In these experiments, co-immunoprecipitation does not provide rigorous evidence that the p125 subunit directly interacts with PCNA, as it is possible that the p125 that is detected by co-immunoprecipitation is by virtue of an interaction of PCNA with a pol δ heterodimer, *i.e.,* the interactions are mediated by a third polypeptide(s). In order to provide a more rigorous test, Sf9 cells were co-infected with baculovirus vectors for p125 and for PCNA. The Sf9 cell lysates were then subjected to gel filtration on a Sephacryl S-300 column (Fig. 2). Assays for pol δ activity showed that there were two peaks of activity, one with a relative molecular weight of 125,000 and the second with a relative molecular weight of 220,000 (Fig. 2, *upper* and *middle panels*). The fractions were analyzed for the presence of p125 and PCNA by Western blotting using specific antibodies. The results show that the 125,000 peak contained only p125, whereas PCNA co-migrated with p125 in the 220,00 molecular weight fractions. Furthermore, no free PCNA was detected in the range where either the dimer or trimer form would be expected to migrate. (In previous studies of the behavior of recombinant PCNA expressed in *E. coli,* we had shown that PCNA in solution is a mixture of dimers and trimers (5)). These results provide the first direct demonstration that free p125 forms a physical complex with PCNA, and moreover, the apparent molecular weight is consistent with a complex of p125 with a trimeric form of PCNA, because previous work showed that the PCNA trimer migrates with an apparent molecular weight of approximately 100,000 on gel filtration (5).

In order to establish that the co-elution of p125 and PCNA is not due to fortuitous associations with unrelated proteins, cross-linking experiments using bifunctional cross-linking

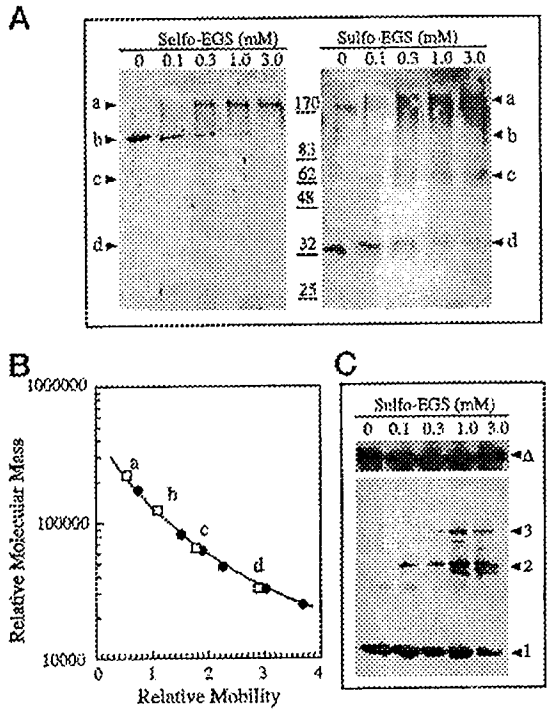


FIG. 3. **Cross-linking analysis of the p125/PCNA complex.** Samples of the peak fraction number 66, (40 μl, 20 ng of protein) from the S-300 chromatography (Fig. 3) were incubated with sulfo-EGS at the indicated concentrations (mM) for 15 min at room temperature and then subjected to SDS-PAGE followed by Western blotting using a monoclonal antibody against p125 (*A, left panel*) and a monoclonal antibody against PCNA (*A, right panel*). *Arrows a* and *b* show p125 immunoreactive bands of 225 and 125 kDa, respectively; *arrow c* corresponds to the cross-linked PCNA dimer, and *arrow d* corresponds to the PCNA monomer. *B* shows the determination of the relative molecular masses of the *bands a–d* (*open squares*); prestained protein standards (New England Biolabs. Inc.) were used as markers (*solid circles*). *C*, a similar cross-linking experiment was performed using a deletion mutant of p125 (Δ2–249) in which the N-terminal 249 residues were deleted (13). The *upper panel* shows the Western blot of the 97 kDa immunoreactive p125 (Δ2–249) (Δ), which was not depleted during the course of the experiment. In addition, no significant formation of higher molecular weight species was observed (not shown). The *lower panel* shows the immunoblot with antibody against PCNA, where *1–3* show the positions of the monomer, dimer, and trimer forms of PCNA, respectively, without evidence for formation of cross-links with p125 (Δ2–249).

agents were performed to establish a direct p125-PCNA protein-protein interaction in the 220,000 molecular weight complex. Sulfo-EGS was used, as we have previously shown that EGS readily cross-links PCNA (5). When the peak fractions of the complex of p125 and PCNA obtained on gel filtration were cross-linked with sulfo-EGS, and the formation of cross-linked species monitored by Western blotting with antibodies against p125 (Fig. 3*A, left panel*) or PCNA (Fig. 3*A, right panel*), a rapid disappearance of the PCNA monomer and p125 was observed with the concomitant formation of a band of 225 kDa that reacted with both p125 and PCNA antibodies. This is consistent with the presence of p125 in complex with a PCNA trimer. It is noteworthy that no accumulation of species that might represent p125 cross-linked to a PCNA monomer or a PCNA dimer was observed.

Control cross-linking experiments with Sf9 cell lysates expressing PCNA or p125 alone were performed. In the case of p125, no cross-linking with sulfo-EGS was observed under the conditions used (not shown). In the case of PCNA, sulfo-EGS gave exactly the same pattern of cross-linking that we had previously found for PCNA cross-linked with EGS (5), in that the major species of PCNA that were observed after cross-linking were the monomer, the dimer, and a smaller amount of the trimer.

In a parallel experiment, a deletion mutant of p125 (Δ2–249) in which the N-terminal 248 residues were removed (13, 20) was co-expressed with PCNA and subjected to cross-linking. In this case, only the monomer, dimer, and trimer species of PCNA were observed, as for PCNA alone, as shown in Fig. 3*C*. Blotting with pol δ antibodies also showed that there were no cross-links formed with the N-terminal deletion mutant of pol δ (Fig. 3*C, upper panel*). These results are consistent with our previous observations that the N-terminal region of p125 is required for its interaction with PCNA.

Far Western Blotting with PCNA—In order to obtain additional evidence that p125 can interact with PCNA, an overlay technique for blotting of PCNA-binding proteins was used. PCNA was labeled with biotin as described under "Experimental Procedures." Pol δ was subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were then blotted with biotinylated PCNA (see under "Experimental Procedures") and visualized using a chemiluminescence method. A number of experiments were performed; they showed that only the p125 band provided a strong reaction with biotinylated PCNA. When immunoaffinity purified calf thymus pol δ enzyme was blotted with biotinylated PCNA, a strong reaction was seen with the p125 catalytic subunit (Fig. 4*A, left panel*). The p50 subunit of pol δ did not interact with PCNA in the overlay experiments (Fig. 4*A, left panel*). The inability of the p50 subunit to interact with PCNA was confirmed using purified recombinant p50 subunit (Fig. 4*A, left panel*). This experiment was repeated using recombinant pol δ heterodimer produced by overexpression of p125 and p50 in Sf9 cells. The same results were obtained, namely that p125 but not p50 reacted with biotinylated PCNA (Fig. 4*B*). Because the overlay depends on an interaction with a polypeptide band separated by SDS-PAGE, this positive interaction demonstrates that the p125 subunit interacts with PCNA in a manner that is independent of the presence of the other subunits of pol δ. In Fig. 4*A*, it is noted that the overlay of immunoaffinity purified pol δ with PCNA reveals a doublet of 70 kDa. In other studies, partial protein sequence was obtained of this band, and a BLAST search identified this polypeptide as KIAA0039 (GenBank™). This was found to be a mammalian counterpart of *S. pombe* Cdc27.[2]

We have also examined the Δ2–249 N-terminal deletion mutant of p125, in which the N2 region is absent (13), by PCNA overlay. The results (Fig. 5*A*) show that this deletion mutant is not recognized by overlay with biotinylated PCNA. In parallel, experiments were performed using deletion mutants Δ186–321, Δ336–715 (core region deleted), Δ675–1107, and Δ778–1047 (C-terminal regions deleted) expressed in Sf9 cells (Fig. 5*B*). All of these deletion mutants, covering essentially the entire p125 sequence from residue 186 (Fig. 5*C*), interacted with PCNA. These results, taken with the inability of the Δ2–249 deletion mutant to bind to biotinylated PCNA, restricts the binding region on p125 to within the first 186 residues.

Yeast Two Hybrid Assay—Human p125, p50 and PCNA coding sequences were inserted into the pAS2–1 and pACT2 vectors and tested for pairwise interactions using the yeast two hybrid system. For these experiments, the yeast co-transformants were grown and the lysates tested for β-galactosidase activity. The results were compared on the basis of relative specific activities (arbitrary units of β-galactosidase activity per unit of cell culture density). The results are shown in Fig. 6. This analysis confirms the biochemical data that p125 and PCNA interact and that p50 and PCNA do not interact.

The N2 Peptide Binds to PCNA—Previous work had identi-

[2] J.-Y. Mo and M. Y. W. T. Lee, submitted for publication.

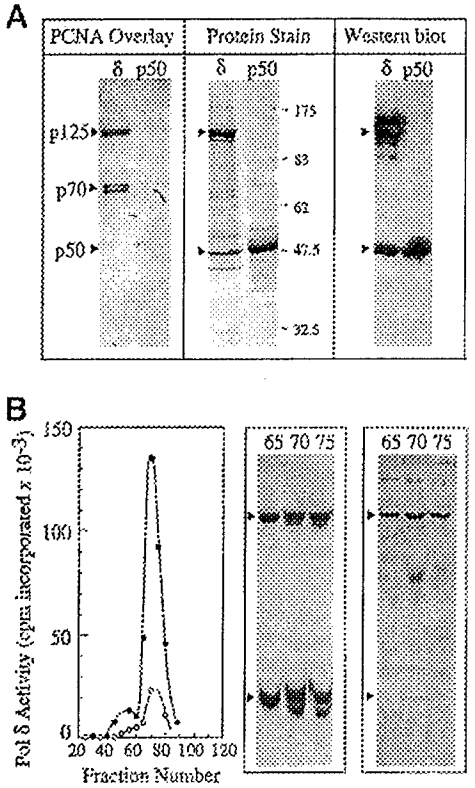


FIG. 4. **PCNA overlay analysis of pol δ.** PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (see under "Experimental Procedures"). Samples were run on SDS-PAGE gels (10% acrylamide), transferred to nitrocellulose, and then blotted using biotinylated PCNA/streptavidin-horseradish peroxidase conjugate. The blots were visualized using a chemiluminescence method (ECL detection system, Amersham Pharmacia Biotech). *A,* the *left panel* shows an experiment using purified calf thymus pol δ obtained by immunoaffinity affinity chromatography (21) that had been further purified on heparin-agarose. This enzyme (δ) was analyzed by PCNA overlay together with recombinant p50 (*p50*) overexpressed in *E. coli* (16) and purified to near-homogeneity (2.5 μg of protein were used). The positions of the p125 and p50 subunits of pol δ are shown by the *arrowheads.* Also shown is the position of a p70 polypeptide that interacts with PCNA. The *center panel* shows the Coomassie Blue-stained SDS-PAGE gel of the same preparations, and the *right panel* is a Western blot using a mixture of monoclonal antibodies to p125 and p50 to show the identity of the polypeptide bands. *B,* p125 and p50 were overexpressed in Sf9 cells and purified to near homogeneity (P. Zhang and M. Y. W. T. Lee, unpublished data). The *left panel* shows the elution of pol δ activity from the final Mono-Q chromatography step, assayed in the absence (○) and presence (●) of PCNA. The *center panel* shows a Western blot of the peak fractions 65, 70, and 75 with a mixture of antibodies to the p125 and p50 subunits. The *right panel* shows the overlay blot with biotinylated PCNA. The *arrowheads* show the migration positions of p125 and p50 subunits.

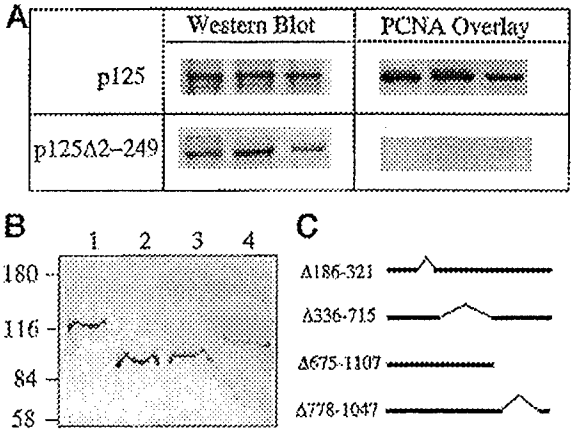


FIG. 5. **PCNA overlay of recombinant p125 and its N-terminal deletion mutants.** *A,* p125 and its N-terminal deletion mutant (Δ2–249) in which residues 2–249 are deleted were expressed in Sf9 cells and analyzed by PCNA overlay. The three *lanes* are the three peak fractions of the recombinant proteins during high pressure liquid chromatography gel filtration that were subjected to SDS-PAGE and overlay analysis with biotinylated PCNA. The *left panels* show the Western blots of the fractions for p125 and the 97 kDa Δ2–249 deletion mutant. The *right panels* show the overlay with biotinylated PCNA. *B,* Sf9 cells (1×10^8) were infected with recombinant baculovirus vectors for Δ186–321, Δ336–715, Δ675–1107, or Δ778–1047. Cell lysates (50 μl) from each of the cultures were then subjected to SDS-PAGE and analyzed by overlay with PCNA as for *A. Lanes 1–4,* represent Δ186–321, Δ336–715, Δ675–1107, and Δ778–1047, respectively. *C,* map of the deletion mutants Δ186–321, Δ336–715, Δ675–1107, and Δ778–1047.

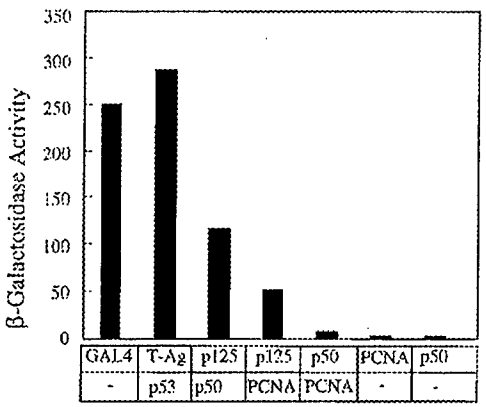


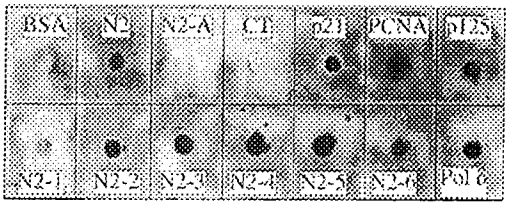
FIG. 6. **Liquid assay for yeast two hybrid interactions between p125 and PCNA.** The p125, p50, and PCNA sequences were tested for pairwise interactions in the yeast two hybrid system as described under "Experimental Procedures." The data are shown as arbitrary units of β-galactosidase activity per unit of cell culture density. T-antigen and p53 constructs were used as controls for a known pair of interacting proteins. The following pairs were tested: p125-p50, p125-PCNA, and p50-PCNA.

fied a region of the N terminus of pol δ, the N2 region (GVTDEGFSVCCHIHGFAPYFY, residues 129–149) as being involved in the interaction of pol δ with PCNA. This was based on the ability of a synthetic peptide with this sequence to inhibit the PCNA stimulation of pol δ (10). However, these experiments were performed with purified pol δ, and in the context of evidence that p125 does not interact with PCNA in other systems, it could be argued that these findings were due to an interference of the peptide with an interaction between p125 and an intermediary protein that leads to loss of PCNA response. Experiments were performed to test for a direct interaction of PCNA with the N2 peptide as well as with several variants with selected alanine replacements by dot blot analysis using biotinylated PCNA (Fig. 7). The peptides were bound to nitrocellulose and then blotted with biotinylated PCNA. The results (Fig. 7) show that immobilized N2 peptides, but not the mutant N2 peptide in which the three terminal YFY residues were replaced with alanine (GVTDEGFSVCCHIHGFAP**AAA**), are blotted by biotinylated PCNA. p21 peptide and p125 were also blotted in this dot blot assay (Fig. 7). The loss of interaction of the mutant in which the three terminal aromatic residues were changed to alanine is highly significant because it provides supportive evidence that the N2 region contains a variant of the PCNA binding motif (see under "Discussion").

DISCUSSION

Biochemical evidence for a direct interaction of the p125 catalytic subunit with PCNA was obtained. The demonstration of a physical complex between recombinant p125 and PCNA by gel filtration and chemical cross-linking with sulfo-EGS provides rigorous evidence for this interaction. In addition, the use of biotinylated PCNA in overlay experiments also shows that

this interaction is not dependent on the presence of other pol δ subunits, and the use of deletion mutants of p125 restricts the location of the interaction site to the N-terminal 186 residues of p125. Further evidence for a region on p125 that is involved in the interaction with PCNA was obtained by the use of synthetic peptides to the N2 region (residues 129–149). These findings confirm and extend previous studies from this laboratory that show that p125 directly interacts with pol δ and that the N2 region in p125 can be implicated in the protein-protein interaction with PCNA. The need for a more detailed investigation of whether there is a direct interaction between p125 and PCNA was raised by studies of pol δ-PCNA interactions in yeast, in which only negative evidence for an interaction of the p125 and PCNA has been obtained. Tratner *et al.* (18) reported that recombinant *S. pombe* p125 did not interact with hemagglutinin epitope-tagged PCNA when they were co-expressed in insect cells and tested for co-immunoprecipitation of ^{35}S-labeled proteins or when tested for pairwise interactions by the yeast two hybrid system. In *S. cerevisiae*, a third subunit of pol δ encoded by the POL32 gene has been identified (22, 23). A ^{32}P-tagged PCNA containing a fused PKA site at the N terminus was used in overlay experiments against p125 (POL3), p58 (POL31), and p55 (POL32) subunits. The results showed that only the p55 subunit bound to the tagged PCNA and no evidence of interaction of either p58 (the p50 homologue) or p125 with PCNA was found (19). The different results that were obtained in the yeast system could be due to differences in the experimental conditions used, as noted by Eissenberg *et al.* (19). The present studies confirm that, unlike the yeast proteins, human PCNA and p125 co-immunoprecipitate with either p125 or PCNA antibodies when they are co-expressed in Sf9 cells, COS 7 cells, and are also co-immunoprecipitated from calf thymus or HeLa extracts. As already noted, co-immunoprecipitation methods do not eliminate the possibility that positive results are due to the intervention of an intermediary polypeptide(s).



N2 GVTDEGFSVCCHIHGFAPYFY
N2-A GVTDEGFSVCCHIHGFAP**AAA**
N2-1 GVTDEG**A**SVCCHIHGFAPYFY
N2-2 GVTDEGF**R**VCCHIHGFAPYFY
N2-3 GVTDEGFSV**A**CHIHGFAPYFY
N2-4 GVTDEGFSVCC**A**IHGFAPYFY
N2-5 GVTDEGFSVCCHI**A**GFAPYFY
N2-6 GVTDEGFSV**AA**HIHGFAPYFY
CT DLEDQEQLLRRFGPPGPEAN
p21 GRKRRQTSMTDFY
HSKRRLIFS

FIG. 7. **Dot blot analysis of binding of PCNA using biotinylated PCNA.** Synthetic peptides/proteins were dot blotted onto nitrocellulose membranes and tested for binding to PCNA using biotinylated PCNA as described under "Experimental Procedures." The proteins tested were bovine serum albumin (*BSA*), recombinant p125 subunit expressed in Sf9 cells (*p125*), and immunoaffinity purified calf thymus pol δ (*pol δ*). The synthetic peptides that were tested are listed below the blot. *CT* is a peptide to the C terminus of p125 (residues 1091–1107) that we have used for the preparation of monoclonal antibody 38B5. *p21* is the p21-derived peptide (139–160) that contains the consensus PCNA binding motif (*underlined*).

An important result obtained in these studies is the first biochemical demonstration of a complex between free recombinant p125 and PCNA by gel filtration. All the gel filtration studies were performed in the presence of 150 mM NaCl, a standard biochemical practice to avoid nonspecific associations. Thus the association between p125 and PCNA takes place at ionic strengths that are near physiological. The cross-linking of the complex with sulfo-EGS showed that there was a rapid cross-linking to a high molecular weight species of a size that was consistent with a p125-PCNA trimer. These results must be taken in the context that under the conditions used we have reproducibly found that PCNA itself is only slowly cross-linked to the trimer and that the bulk of the reaction products are the monomer and dimer species (5). Previous observations from this laboratory have shown that PCNA in solution is an equilibrium mixture of the dimer and trimer species (5). The findings that the only complex present was a PCNA trimer-p125 complex, with an absence of dimeric or monomeric PCNA complexes with p125, suggests that p125 either selectively binds to the PCNA trimeric form or stabilizes the PCNA trimer. This preference for trimeric PCNA is consistent with the physiologically expected interaction of pol δ with PCNA, the function of which is associated with a trimeric state. It is also consistent with current models of the assembly of the replication complex,

TABLE I
Alignment of the N2 region of pol δ with the PCNA binding motif

			PCNA Binding Motif	Gen Bank no.
			12345678 901234567	
Pol δ	*S. cerevisiae*	151	CNV TGFKNYL**Y** VPAPNSSDA	X61920
Pol δ	*S. pombe*	130	VHV VGF**L**PY**FY** VKAPVGFRP	LO7734
Pol δ	Human	139	CHI HGFAPY**FY** TPAPPGFGP	M81735
UDG	Human	1	MIG QKTLYS**FF** SPSPA**R**K**R**H	X89398
MCMT	Human	158	STR QTTITSH**F** AKGPA**K**R**K**P	X63692
Tigger Cds2	Human	86	LMR QTSLLS**YF** **K**KLPQPPQP	U49973
Rad2	*S. pombe*	335	TIP QGRLDS**FF** KPVPSSP**KK**	X77071
RAD27	*S. cerevisiae*	337	SGI QGRLDG**FF** QVVPKTKEQ	P26793
DNA ligase I	Human	1	M QRSIMS**FF** HPKKEGKAK	M36067
p57	Human	266	KLS GPLISD**FF** ATRKRSAPE	U48869
p21$^{Waf1/Cip1}$	Human	141	KRR QTSMTD**FY** HSKRRLIFS	P38936
p21	*Drosophila*	181	RKR QPKITE**FM** KERKRAAQA	U68477
POL32	*S. cerevisiae*	335	LKK QGTLES**FF** KRKAK*	Z49543
UDG	*S. cerevisiae*	18	KRK QTTQED**FF** GTKKSTNEA	J04470
Cdc27	*S. pombe*	359	KPQ QKSIMS**FF** GKK*	M74062
POGO	*Drosophila*	489	AVL QKKITD**YF***	S20478
FEN1	Human	334	GST QGRLDD**FF** KVTGSLSSA	P39748
XPG	Human	987	QQT QLRIDS**FF** KLAQQEKED	P28715

UDG, uridine DNA glycosylase; MCMT, methyl 5′ cytosine DNA methyl transferase. The eight residue PCNA binding motif is numbered 1–8 at the top. Conserved residues are in bold at positions 1, 4, 7, and 8. In the C-terminal regions (numbered 9–17), proline residues are underlined and basic residues (arginine and lysine) are in bold.

in which replication factor C first loads PCNA onto DNA, following which pol δ is recruited (8). Recent findings (24) have shown that replication factor C disengages from PCNA upon sliding clamp formation and that the loaded PCNA clamp was able to recruit and bind polymerase δ and stimulate DNA replication.

Recently, a number of additional proteins that bind to PCNA have been identified (4, 9, 11, 25). These findings have major implications for an understanding the roles of PCNA. These PCNA-binding proteins fall into three major groups: DNA replication proteins (pol δ, replication factor C and pol ϵ, flap endonuclease 1, and DNA ligase), DNA repair proteins (xeroderma pigmentosum G and methyl(5-cytosine)methyl transferase), and cell cycle regulatory proteins (p21 and p57). These proteins contain a short PCNA binding motif in which there is a conserved glutamine and two conserved aromatic residues. The motif in question lies at the N terminus of the p21 peptide that forms an α-helical region in which the two aromatic residues interact with the large hydrophobic pocket of PCNA, whereas the C-terminal region forms an anti-parallel β sheet with the interdomain connector loop of PCNA (7).

An obvious question that arises is whether the N2 region of the p125 subunit of pol δ harbors a sequence that corresponds to the PCNA binding motif that has been identified in these PCNA-binding proteins. The alignment of the N2 sequence of pol δ from human and yeast with the PCNA binding motifs of a number of proteins is shown in Table I, which shows the eight-residue motif bounded by the conserved glutamine and the aromatic residues. The main features of the motif are the conserved glutamine at position 1, the presence of an aliphatic residue (leucine or isoleucine) at position 4, and a pair of aromatic residues at positions 7 and 8. Comparison of the N2 sequence shows that it possesses the aromatic residues and the aliphatic residue at position 4, but does not have the conserved glutamine. Thus, this region of the N2 sequence does not carry a complete consensus with the known PCNA binding motif. However, this glutamine residue is not conserved in p57, which also binds PCNA (Table I). Evidence obtained in this study using peptides to the N2 region of pol δ provides strong evidence that it contains a variant of the PCNA binding motif (Table I). The loss of binding when the aromatic residues are mutated in the N2 peptide does provide significant evidence that this region of the N2 sequence may be a member of the PCNA binding motif family of sequences.

In Table I, the regions C-terminal to the PCNA binding motif are also aligned. In the case of p21, this region is involved in the second major protein-protein contact of the peptide with PCNA and forms an anti-parallel β-sheet with the interdomain connector loop of PCNA (7). In previous studies, it had been shown that the interdomain connector loop of PCNA is important for the interaction of pol δ, and it was proposed that the N2 region of pol δ may interact with PCNA in a manner that may be similar to the interaction of p21 with PCNA (26, 27). There is a group of PCNA-binding proteins in which there is a preponderance of basic residues in this region (Table I), as previously noted by Warbrick *et al.* (28). However, it is also seen that there is a second group of sequences, in which there is a high percentage of proline residues. This includes pol δ from human and yeasts, human uracil DNA glycosylase, methyl 5′-cytosine methyl transferase, the human Tigger sequences (28), and *S. pombe* RAD2. The positions of the prolines is well conserved in these eight examples (Table I). The presence of this "proline-rich" motif provides additional support for the view that the N2 sequence belongs to the family of PCNA binding sequences. Furthermore, it should not be forgotten that from the example of p21 that the *QXXI/LXXFF* motif reflects an interaction only with the large hydrophobic pocket of PCNA. Although examples also exist that indicate that this interaction alone (*Drosophila* POGO and *S. pombe* Cdc27) is sufficient for PCNA binding, the presence of the two features of the C-terminal region in the form of the proline rich motif and the basic motif suggests that these may represent sequences that are suited to the formation of a β-sheet with the interdomain connector loop of PCNA.

In summary, a detailed approach was undertaken to investigate the issue of whether the p125 polypeptide directly interacts with PCNA. Our findings provide strong biochemical confirmation that there is a direct interaction between the p125 subunit and PCNA, although they do not eliminate the possibility that the pol δ holoenzyme has multiple sites of interaction with PCNA through one or more of its subunits. The latter possibility is one that could be facilitated by the trimeric nature of PCNA, because this provides for extended interactions of individual PCNA subunits with different subunits of pol δ.

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Evidence That DNA Polymerase δ Isolated by Immunoaffinity Chromatography Exhibits High-Molecular Weight Characteristics and Is Associated with the KIAA0039 Protein and RPA†

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ABSTRACT: DNA polymerase δ, the key enzyme for eukaryotic chromosomal replication, has been well characterized as consisting of a core enzyme of a 125 kDa catalytic subunit and a smaller 50 kDa subunit. However, less is known about the other proteins that may comprise additional subunits or participate in the macromolecular protein complex that is involved in chromosomal DNA replication. In this study, the properties of calf thymus pol δ preparations isolated by immunoaffinity chromatography were investigated. It is demonstrated for the first time using highly purified preparations that the pol δ heterodimer is associated with other polypeptides in high-molecular weight species that range from 260000 to >500000 in size, as determined by FPLC gel filtration. These preparations are associated with polypeptides of ca. 68−70, 34, 32, and 25 kDa. Similar findings were revealed with glycerol gradient ultracentrifugation. The p68 polypeptide was shown to be a PCNA binding protein by overlay methods with biotinylated PCNA. Protein sequencing of the p68, p34, and p25 polypeptide bands revealed sequences that correspond to the hypothetical protein KIAA0039. KIAA0039 displays a small but significant degree of homology to *Schizosaccharomyces pombe* Cdc27, which, like *Saccharomyces cerevisiae* Pol32p, has been described as the third subunit of yeast pol δ. These studies provide evidence that p68 is a subunit of pol δ. In addition, the p68−70 and p32 polypeptides were found to be derived from the 70 and 32 kDa subunits of RPA, respectively.

DNA replication is a vital cellular process in which the basic synthetic reactions are performed by the DNA polymerase enzymes. These enzymes are the central components of larger assemblies of proteins that are required for cellular DNA replication. DNA polymerase δ, the key enzyme for eukaryotic chromosomal replication, has been well characterized as consisting of a core enzyme of a 125 kDa catalytic subunit and a smaller 50 kDa subunit. In *Escherichia coli*, the DNA polymerase III holoenzyme consists of at least 10 different polypeptides (*1*, *2*). Biochemical and genetic studies have enabled the formulation of models in which these form a multiprotein assembly that functions to coordinate both leading and lagging strand DNA synthesis at the replication fork (*2*). This includes a mechanism whereby this complex contains two DNA polymerase molecules linked by a dimerization protein (tau). This model has been proposed for T4 and *E. coli* as a means of concurrent replication of both template strands at the replication fork (*3*−*5*). Similar models have been proposed for eukaryotic systems, although the details of the proteins or their macromolecular assembly are still vague (*6*).

DNA polymerase δ is the main replicative polymerase involved in the duplication of eukaryotic cell chromosomal DNA (*7*−*11*). Despite the central importance of this enzyme, the delineation of its subunit structure to this date is far from complete. Rigorously purified mammalian pol δ has been extensively characterized as a tightly associated heterodimer consisting of a 125 kDa catalytic subunit and a small 50 kDa subunit (*12*−*14*). The pol δ catalytic subunit is highly conserved in eukaryotic cells, e.g., between human and yeast (*15*). In recent work, *Schizosaccharomyces pombe* pol δ has been isolated in a form that contains two additional subunits (*16*), while in *Saccharomyces cerevisiae*, pol δ has been shown to consist of three subunits (*17*). In addition, the recombinant *S. cerevisiae* pol δ heterotrimer can dimerize when analyzed on Superose 6 gel filtration columns (*18*). This third subunit is encoded by the POL32 and Cdc27 genes in *S. cerevisiae* and *S. pombe*, respectively (*17*, *19*), while the fourth subunit, only identified in *S. pombe*, is encoded by the Cdm1 gene (*16*, *19*).

The task of identifying new subunits of mammalian DNA polymerases is made difficult by the low amounts of enzyme in mammalian tissues and the instability of the enzyme (*12*, *13*). Very little is known of the polypeptides that are associated with mammalian pol δ at the level of the demonstration of physical complexes that are more highly organized than the heterodimer. In an effort to facilitate the identification of proteins and enzymes that interact with pol δ and PCNA, we have developed PCNA affinity (*20*) and

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pol δ immunoaffinity chromatography methods (*14*). A pol δ heterodimer consisting of p125 and p50 was readily obtained when immunoaffinity-purified pol δ is subjected to single-stranded DNA cellulose chromatography (*14*). Using a PCNA overlay technique, we have identified another subunit of polymerase δ, p68. A partial protein sequence and a BLAST search identified this polypeptide as KIAA0039 (*21*). In this study, we have investigated the behavior of the immunoaffinity-purified pol δ from calf thymus, and demonstrated that it exhibits the behavior of a higher-order complex with other polypeptides at a high level of purification.

EXPERIMENTAL PROCEDURES

Materials. Single-stranded DNA cellulose and heparin−agarose were obtained from Sigma Chemical Co. Hydrazide Avid gel F was from Unisyn Technologies. Poly(dA)$_{2000}$ was obtained from Midland Certificate Co. Superose 12 columns, a protein biotinylation system, and the ECL chemiluminescence detection reagents were purchased from Amersham-Pharmacia Biotech Inc. Fetal calf thymus glands were obtained from Animal Technologies Inc. The lysis buffer used for homogenization of calf thymus consisted of 50 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, and 5% glycerol. TGEED buffer consists of 50 mM Tris-HCl (pH 7.8 or 8.5), 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol. TGEE buffer is the same as TGEED buffer except for the omission of dithiothreitol. KGEED buffer is 20 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.

Purification of DNA Polymerase δ from Calf Thymus. All steps were carried out at 0−4 °C using the procedures described by Jiang at al. (*14*), unless otherwise indicated. Eight hundred grams of frozen calf thymus tissue in 4 L of lysis buffer was homogenized in a Waring blender. The suspension was centrifuged at 5000 rpm at 4 °C for 1 h. The supernatant was filtered through glass wool.

Batchwise DEAE-Cellulose Adsorption. DE-52 cellulose (1.5 L, Whatman) was equilibrated with TGEED buffer (pH 7.8). The supernatant was mixed with the DE-52 cellulose and stirred for 30 min. The mixture was filtered through a Buchner funnel. The DE-52 cellulose was washed with 10 L of TGEED (pH 7.8), and the pol δ was stripped with 3 L of 20% ammonium sulfate in TGEED (pH 7.8).

Phenyl−Agarose Hydrophobic Chromatography. The DE-52 cellulose fraction was loaded onto a phenyl−agarose column (bed volume of 500 mL) equilibrated with KGEED buffer [20% ammonium sulfate (pH 7.0)]. The phenyl−agarose column was washed with 1 L of KGEED and eluted with 1 L of TGEED (pH 8.5).

Immunoaffinity Chromatography. The peak fractions from step 2 were pooled and precipitated by addition of 0.32 g/mL ammonium sulfate. The suspension was stirred for 30 min and kept on ice for an additional 30 min, and then centrifuged at 10000*g* for 45 min. The precipitate was resuspended in TGEE buffer, and the conductivity was adjusted to that of TGEE buffer with 80 mM NaCl. The solution was divided into two equal batches, which were individually subjected to immunoaffinity chromatography. The immunoaffinity column (bed volume of 20 mL) was equilibrated with TGEE buffer (pH 7.8). After the sample had been loaded, the column was washed with 60 mL of TGEE buffer containing 0.4 M NaCl. Pol δ was eluted with 30% ethylene glycol and 0.4 M NaCl in TGEE. The peak fractions from two batches of immunoaffinity chromatography were then combined.

Single-Stranded DNA Cellulose Chromatography. The fractions from the immunoaffinity column were combined, and the conductivity was adjusted to that of TGEED buffer containing 50 mM NaCl and loaded onto a ssDNA cellulose column (bed volume of 20 mL). The column was washed with 50 mL of TGEED buffer (pH 7.8). The enzyme was eluted with a 200 mL gradient from 50 to 700 mM NaCl.

Heparin−Agarose Chromatography. The fractions from the ssDNA cellulose column were combined and the conductivities adjusted to that of TGEED buffer containing 50 mM NaCl, and the fractions were loaded onto a heparin−agarose column (bed volume of 2 mL). The column was washed with TGEED buffer (pH 7.8), and the pol δ was eluted with 0.4 M NaCl in TGEED buffer.

FPLC Gel Filtration Chromatography. A preparation obtained after immunoaffinity chromatography was dialyzed against TGEED buffer (pH 7.8) with two changes over a period of 16 h, and then concentrated to 300 μL by centrifugal concentration (5000*g* at 4 °C) using Centricon 30 filters (30 000 MW cutoff, Millipore). The concentrated pol δ was then chromatographed on a FPLC Superose 12 column (HR 10/30, Pharmacia) equilibrated with TGEED (pH 7.8) containing 150 mM NaCl at a flow rate of 0.25 mL/min. A total of 72 fractions of 0.25 mL each were collected.

Glycerol Gradient Ultracentrifugation. Sedimentation analysis was carried out using a Beckman ultracentrifuge with a SW 41 rotor. The buffer was TGEED (pH 7.8) with 150 mM NaCl. The proteins were laid on the top of glycerol gradients in 12 mL tubes and centrifuged at 30 000 rpm for 16 h. After centrifugation, fractions were withdrawn from the bottom of the tubes. The sedimentation velocities were estimated by the use of standard proteins.

SDS−Polyacrylamide Gel Electrophoresis. Fractions were precipitated with 10% trichloroacetic acid, washed with cold 80% acetone, and dissolved in SDS loading buffer. The samples were loaded on a 10% SDS−polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue or silver (Bio-Rad).

Western Blotting. Western blotting was performed using 78F5 and 38B5 pol δ monoclonal antibodies (*14*). Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and also to provide for visual confirmation of efficient transfer. Nitrocellulose blots were blocked in 5% w/v nonfat dry milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% (v/v) Tween 20 (TBST) for 1 h at room temperature. The blot was then incubated with monoclonal antibodies against pol δ for 12 h at 4 °C. After three 10 min washes in TBST, the blot was incubated with the streptavidin−horseradish peroxidase conjugate diluted in TBST (1:10000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence (ECL detection system, Amersham-Pharmacia Biotech Inc.).

Nondenaturing Polyacrylamide Gel Electrophoresis. The samples were run on a 5 to 15% gradient gel with a 3.5% stacking gel at 4 °C for a sufficient length of time such that all the protein markers and the pol δ band had reached limiting mobilities. An 18 h period of electrophoresis at 200 V was found to be suitable in preliminary trial experiments. SDS and 2-mercaptoethanol were excluded from the gel. In this experiment, a crude calf thymus extract which has been subjected to batchwise purification on DEAE-cellulose was analyzed. The DEAE sample was concentrated via ammonium sulfate precipitation, and the sample was subsequently desalted on a desalting column. The sample (1 mg of protein) was electrophoresed for 18 h at 4 °C along with the protein standards. Proteins were transferred to a nitrocellulose membrane at 12 V for 12−14 h at 4 °C. The membranes were immunoblotted with 78F5 pol δ p125 monoclonal antibody.

Overlay Blotting with Biotinylated PCNA. Recombinant PCNA was expressed in *E. coli* and purified to near homogeneity as previously described (*22*). PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (Amersham-Pharmacia Biotech Inc.). The reaction mixtures contained 0.5 mg of PCNA and 0.5 mg/mL biotinamidocaproate *N*-hydroxysuccinamide ester in a total volume of 0.5 mL in 20 mM bicarbonate buffer (pH 8.6). After reaction for 60 min at room temperature, the PCNA was purified on a Sephadex G25 column (bed volume of 5 mL) equilibrated with phosphate-buffered saline containing 1% bovine serum albumin. Samples (1−5 μg of protein) to be overlayed were run onto a 10% SDS−PAGE gel and transferred to nitrocellulose membranes. Prestained protein standards (New England Biolabs) were used as molecular weight markers. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBST for 45 min at room temperature followed by three washes of TBST for 10 min each. The blot was then incubated with biotinylated PCNA (1 μg/μL) diluted in TBST (1:900) at 4 °C overnight. The blot was washed five times with TBST for 15 min. It was subsequently incubated with the streptavidin−horseradish peroxidase conjugate diluted in TBST (1:5000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence.

DNA Polymerase Assay. Sparsely primed poly(dA)$_{2000}$/oligo(dT)$_{16}$ was used as the template. The assays contained poly(dA)$_{2000}$/oligo(dT)$_{16}$ (20:1, 0.25 OD$_{260}$ unit/mL), 200 μg/mL BSA, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES (pH 6.0), 20 μM [^{3}H]TTP (100 cpm/pmol, 5 μCi/nmol), and 0.2−0.4 unit of pol δ, in the presence or absence of 0.2 μg of PCNA in a total volume of 100 μL. When poly[d(AT)] was used as a template, assays were performed in the absence of PCNA as described by Lee et al. (*13*). The reaction mixtures were incubated for 60 min at 37 °C, and the reactions were terminated by spotting onto DE-81 papers which had been washed four times with 0.3 M ammonium formate (pH 7.8) and once with 95% ethanol and counted as previously described (*13*). One unit of DNA polymerase activity is the amount that catalyzes the incorporation of 1 nmol of dTMP per hour at 37 °C.

Protein Quantitation. Protein was quantified by the Bradford method with BSA as a standard (*23*).

Peptide Sequencing. Sequence analyses were performed by the Harvard University Microchemistry Facility using single microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer.

RESULTS

Immunoaffinity-Purified Pol δ Behaves as a High-Molecular Complex That Is Much Larger Than the Heterodimer. A number of methods have been reported for the rigorous isolation of pol δ from mammalian sources by conventional methods. These generally lead to the isolation of a tightly associated heterodimer of 125 and 50 kDa subunits, although there are reports of the isolation of the free p125 catalytic subunit (*24*, *25*). The amounts recovered are generally very small (tens of micrograms). We reported a more facile procedure that involves batchwise purification of pol δ from calf thymus through DE-52 and phenyl−agarose supports, followed by immunoaffinity chromatography using a monoclonal antibody directed against the p125 catalytic subunit (*14*). This procedure yields about 1 mg of protein, which on SDS−PAGE contains the p125 and p50 polypeptides of pol δ but also a number of other polypeptide bands. However, a persistent association of a ca. 70 kDa band was also noticed. Final purification to the heterodimer was achieved by ssDNA cellulose chromatography (*14*). The specific activity of the preparation was similar to that reported previously (*13*, *14*).

The question which arose was whether any of the polypeptide bands found with the pol δ heterodimer after immunoaffinity chromatography represented replication proteins, i.e., whether the immunoaffinity-purified enzyme contains additional pol δ subunits or other replication proteins that contribute to a higher-order complex. FPLC gel filtration analysis was used to determine if the immunoaffinity-purified enzyme behaved as a higher-molecular weight complex(es) than could be ascribed to a heterodimer. To do this, the immunoaffinity-purified enzyme was concentrated before FPLC analysis. This was done as any higher-order complexes were more likely to be dissociated on dilution. The column fractions were assayed for DNA polymerase δ activity using poly(dA)/oligo(dT) as a template in the absence and presence of PCNA. The fractions were also assayed with poly[d(AT)] alternating copolymer as a template in the absence of PCNA as described previously (*13*). The enzyme activity behaved in a polydisperse manner, but the pol δ activity eluted with a molecular weight higher than that found for the heterodimer (M_r = 175 000), as shown in Figure 1. The major peaks of pol δ activity ranged from a relative molecular weight of 230000 to >500000 as determined from calibration of the column with protein standards. In Figure 1, the identities of the 125 and 50 kDa polypeptides as the subunits of pol δ were confirmed by Western blotting. SDS−PAGE of the peak fractions of the high-molecular weight fractions of immunoaffinity-purified pol δ revealed that the preparations contained the 125 and 50 kDa subunits as major components; i.e., these represent very highly purified preparations. Typical SDS−PAGE profiles for the fractions obtained on FPLC gel filtration of the immunoaffinity-purified enzyme are shown for two separate preparations in Figure 2. In addition to the p125 and p50 polypeptides, we consistently observed the presence of a number of other polypeptides. These included a ca. 70 kDa band, which often appeared as a doublet, and

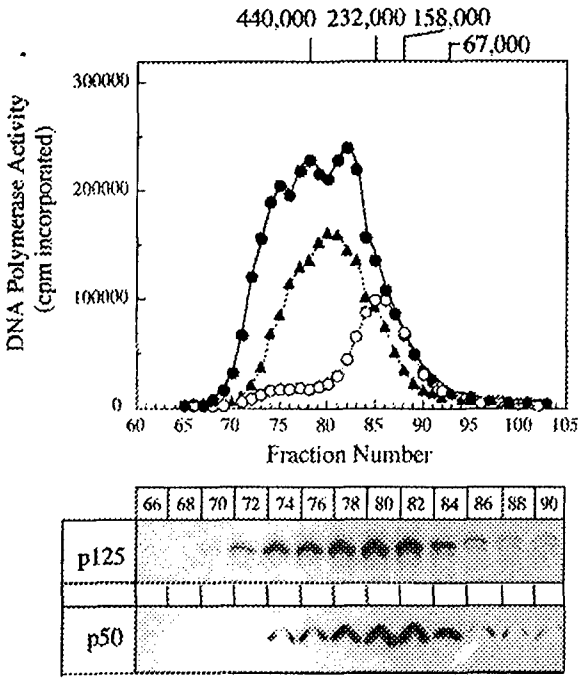

FIGURE 1: FPLC gel filtration analysis of immunoaffinity-purified fetal calf thymus DNA polymerase δ. Calf thymus pol δ was purified by immunoaffinity chromatography and concentrated by centrifugation on Centricon filters as described in Experimental Procedures. The concentrated preparation was run on a Superose 12 HR10/30 column on a Bio-Rad Biologics FPLC system. Fractions of 0.15 mL were collected. The column was calibrated with protein standards (ferritin, catalase, aldolase, and albumin). (Top) Five microliters of each fraction was assayed with poly(dA)/oligo(dT) in the presence (●) and absence (○) of added calf thymus PCNA at 37 °C for 30 min. Activated poly(dAT) was also used as a template (▲) and assayed in the absence of PCNA. (Bottom) Samples of the active fractions were subjected to SDS−PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The membrane was blotted with antibodies against p125 and p50.

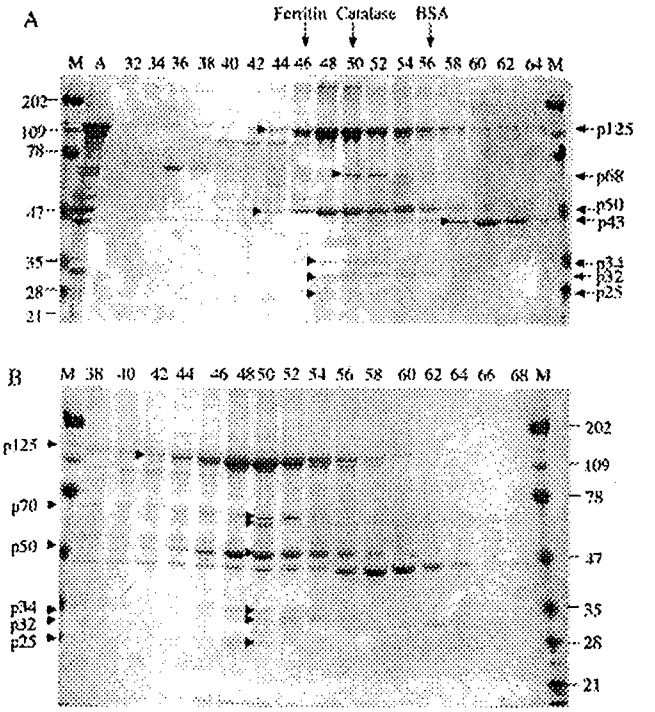

FIGURE 2: SDS−PAGE of immunoaffinity-purified pol δ after FPLC gel filtration. Calf thymus pol δ was purified to the immunoaffinity chromatography step as described for Figure 1, and subjected to FPLC gel filtration using a Superose 12 column. In these experiments, fractions of 0.25 mL were collected. Samples of the active fractions were run on SDS−PAGE and stained with Coomassie Brilliant Blue, and the diagram (A and B) shows the results from two separate representative experiments. Protein standards were as indicated on the left of the diagram and were run in the lanes marked M. A sample of the immunoaffinity-purified enzyme preparation before gel filtration was run in the lane marked A (panel A). The numbers refer to the column fractions which were analyzed. The position of elution of ferritin (MW of 440 000), catalase (232 000), and bovine serum albumin (BSA, 67 000) are shown by the arrows above the gel which indicate the fraction numbers at which these standards eluted.

bands at 43, 34, 32, and 25 kDa. These experiments were repeated for at least 20 preparations, and the appearance of these polypeptides was consistent, although the amounts were variable from preparation to preparation. These bands did not appear in stoichiometric amounts with p125 and p50.

In the following experiments, (a) additional evidence was obtained which shows that the immunoaffinity-purified enzyme behaves as a larger physical entity than can be ascribed to the size of the heterodimer, (b) the identities of the ancillary polypeptides were investigated by protein sequence determination, and (c) the PCNA response of the immunoaffinity-purified enzyme is shown to differ from that of the heterodimer.

Comparison of Physical Properties of the Immunoaffinity-Purified Pol δ with Those of the Heterodimer. The immunoaffinity-purified enzyme was passed through a single-stranded DNA cellulose column and subsequently onto a heparin−agarose column as described in Experimental Procedures, leading to the isolation of a heterodimer of p125 and p50 (not shown) as previously reported (*14*). Calibration of the FPLC gel filtration column on which the immunoaffinity preparations were chromatographed (Figure 2) indicated that the p125 polypeptide (based on SDS−PAGE and protein staining) eluted with a peak between fractions 49 and 50, corresponding to relative molecular weights between 250 000 and 300 000 (not shown). The behavior of the enzyme was not due to aggregation since similar findings were obtained when the amounts of enzyme loaded were reduced 10-fold. Previous studies have shown that the heterodimer behaves as a protein with a relative molecular weight of 173 000 (*13*). This was confirmed with the FPLC gel filtration column used in these studies (not shown). A plot of the Stokes radii of the immunoaffinity-purified enzyme as determined by FPLC chromatography on Superdex 200 gave a value of 57 Å based on the peak fraction containing the p125 band as determined by SDS−PAGE. This is larger than the value of 53 Å that we have previously determined for the heterodimer by conventional purification (*13*).

Similar results were obtained by using glycerol gradient ultracentrifugation (Figure 3); i.e., the immunoaffinity-purified enzyme sedimented with a much higher velocity ($S_{20,w}$ = 9.2) than the heterodimer which under the same experimental conditions migrated as a species of about 7.0 S (not shown). The fractions of pol δ activity obtained after glycerol gradient ultracentifugation were run on SDS−PAGE and stained with silver (Figure 3, inset). It may be noted that p68, p34, p32, and p25 polypeptides also cosedimented with the core enzyme. These experiments demonstrate that the immunoaffinity-purified calf thymus pol δ activity

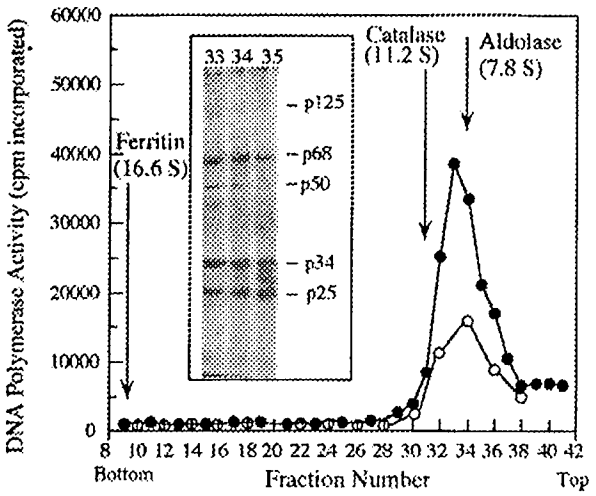

FIGURE 3: Glycerol gradient centrifugation of the immunoaffinity-purified pol δ. Pol δ was purified to the immunoaffinity chromatography step. The peak fractions were concentrated to 150 μL on Centricon filters (Experimental Procedures), laid on the top of a 10 to 45% glycerol gradient (11 mL) containing 150 mM NaCl, 1 mM DTT, and 50 mM Tris-HCl (pH 7.8), and centrifuged for 16 h at 25 000 rpm. After centrifugation, fractions of 0.25 mL each were withdrawn from the bottom of the tubes and assayed for polymerase activity in the presence (●) and absence (○) of PCNA using poly(dA)/oligo(dT) as the template. Migration positions of protein standards (ferritin, catalase, and aldolase) are shown by the arrows. In the inset, the proteins from the peak fractions of activity (fractions 33−35) were concentrated using Centricon 30 filters and loaded onto a 10% SDS−polyacrylamide gel. The gel was visualized by silver staining. The lines show the p125, p68, p50, p34, and p25 polypeptides.

Table 1: Molecular Weights of Mammalian DNA Polymerase Preparations

ref	source	Stokes radius (Å)	sedimentation coefficient	molecular mass
Goulian et al. (*24*)	mouse	54	8.0	178
		43	6.3	112
Lee et al. (*13*)	calf thymus	53	7.9	173
this work	calf thymus	57	9.2	215

behaves as a larger complex than the heterodimer. These findings are the first demonstration that highly purified pol δ in which the core enzyme is the principle component behaves as a macromolecular complex. The calculated molecular weights are shown in Table 1, and are larger than those previously reported for the calf thymus (*13*) and mouse (*24*) pol δ preparations.

Sequence Analysis of Polypeptides in the Pol δ Complex. The identities of the polypeptides that are associated with pol δ preparations were investigated by sequence analysis of the bands excised from the preparations shown in Figure 2, i.e., of the fractions obtained on FPLC gel filtration of immunoaffinity-purified pol δ. A list of the protein sequences obtained from polypeptides excised from SDS−PAGE gels from the two preparations (Figure 2) is shown in Table 2. The p125 and p50 bands were also excised, and the peptide sequences that were obtained exactly matched the known sequences of p125 and p50 (Table 2).

We have previously shown that the doublet of proteins of 68−70 kDa that are associated with high-molecular weight form of affinity-purified pol δ are PCNA binding polypeptides (*21*). Sequencing of bands from this region was performed by MS/MS peptide sequencing, yielding sequences that are identical to the KIAA0039 cDNA sequence (*21*). Additional analysis showed that the peptide sequences obtained from the p68/p70 band (Figure 2 and Table 2) could be arranged into two groups, showing that there were two polypeptides that were migrating in this region. Five sequences (Table 2) were exactly identical with the open reading frame encoded by the human cDNA (Genbank entry D26018) for the hypothetical protein KIAA0039 (*26*). The cDNA encoded by KIAA0039 contains an open reading frame of 466 amino acids. The hypothetical KIAA0039 protein has a predicted molecular mass of 51.4 kDa. The extreme C-terminus contains a consensus PCNA binding site, consistent with the results of the PCNA overlay experiments (*21*). The latter observation makes this protein a strong candidate for the human homologue of the "third" subunits of yeast pol δ, which are encoded by the Cdc27 and POL32 genes in *S. pombe* and *S. cerevisiae*, respectively (*16*, *17*). The second of the groups of sequences obtained from the p68 polypeptide was found to be a match for the 70 kDa subunit of RPA, the eukaryotic single-stranded DNA binding protein.

The p34 and p25 bands also yielded sequences identical to those in the KIAA0039 sequence. Five peptide sequences obtained from the p34 polypeptide, and one peptide sequence obtained for the p25 polypeptide, were found to be derived from KIAA0039. This indicates that these two bands are proteolytic fragments of the p68 full-length KIAA0039 protein. The association of these KIAA0039 fragments with the high-molecular weight fraction of pol δ strongly suggests that they may be associated with nicked species of KIAA0039 which retained an ability to associate with pol δ. This could also explain failure to find a consistent stoichiometric association of KIAA0039 in these particular experiments.

The p43 polypeptide, which is present in the immunoaffinity-purified preparation but is clearly separated from the heterodimer on gel filtration (see Figure 2), was also sequenced and was identified as actin.

PCNA Overlay of Immunoaffinity-Purified Pol δ. We have shown that biotinylated PCNA can be used to identify PCNA binding proteins, and have used this method to demonstrate that PCNA interacts with pol δ p125 (*21*). The p50 subunit does not bind PCNA by this method (*21*). The results of a typical overlay experiment in which pol δ preparations at different stages of purification were examined are shown in Figure 4A. It is seen that there are a number of PCNA binding polypeptides in the crude extract, and that the prominent ones are p125 and a doublet at around 68 kDa. The partial protein sequence of this 68 kDa band was obtained, and a BLAST search identified this polypeptide as KIAA0039 (*21*). This protein is retained in the preparation up to the immunoaffinity step, and is retained with the p125 band during subsequent FPLC gel filtration on Superose 12 (Figure 4A). Thus, highly purified pol δ preparations are associated with a ca. 68 kDa polypeptide which is the mammalian counterpart of *S. pombe* Cdc27. If the immunoaffinity-purified pol δ enzyme is first chromatographed on ssDNA−cellulose, this polypeptide is removed and only the p125 band can be detected by PCNA overlay (Figure 4B).

Native Gel Electrophoresis. The nature of the complex(es) involving the p125 catalytic subunit of pol δ was investigated by Western blot analyses of partially purified pol δ on

Table 2: Sequence Analysis of Polypeptides in Immunoaffinity-Purified Calf Thymus Pol δ[a]

excised polypeptide band (kDa)	amino acid sequence	residue numbers	identity	Genbank accession no.
125	TEGGEDYTGATVIEPLK	574−590	p125 subunit of pol δ	M80395
	GLLPQILENLLSAR	653−666		
50	YIHPDDELVLEDELQR	126−141	p50 subunit of pol δ	U2109
	QAASVEAVKMLDEIL	269−283		
	YSSMEDHLEILEWTL	355−369		
70	VVILMELEVLK	93−103	RPA 70 kDa subunit	M63488
	LFSLELVDESGEIR	221−234		
	NEQAFEEVFQNANFR	554−568		
68	WLSYTLGVHVNQAK	25−38	KIAA0039 protein	D26018
	QMLYDYVER	39−47		
	DSGPLFNTDYDILK	110−123		
	FSAIQCAAAVPR	132−143		
	GIMGMFASK	191−199		
34	WLSYTLGVHVNQAK	25−38	KIAA0039 protein	D26018
	QMLYDYVERK	39−48		
	AMLKDSGPLFNTDYDILK	106−123		
	DSGPLFNTDYDILK	110−123		
	GIMGMFASK	191−199		
32	IGNVEISQVTIVGIIR	66−81	RPA 32kDa subunit	gi|4506585|
	IDDMTAAPMDVR	94−105		
	KSLVAFK	139−145		
	PRGLNFQDLK	222−231		
	NQLKHMSVSSIK	232−243		
25	DSGPLFNTDYDILK	110−123	KIAA0039 protein	D26018

[a] Protein sequences from polypeptides extracted after SDS−PAGE were obtained as described in Experimental Procedures.

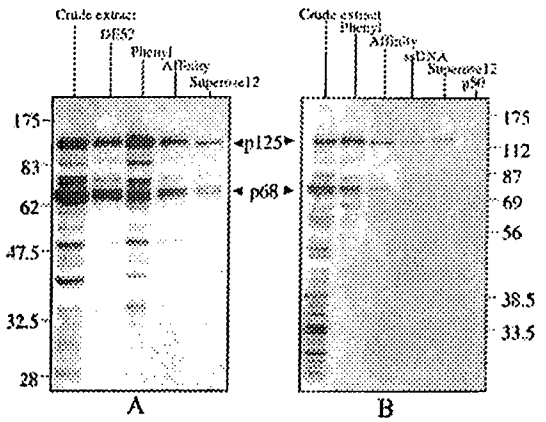

FIGURE 4: PCNA overlay analysis of DNA pol δ at different stages of purification. (A) Samples of a pol δ preparation from the crude extract, DE-52, phenyl−agarose, immunoaffinity, and Superose 12 gel filtration steps were run on SDS−PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The membrane was blotted using biotinylated recombinant PCNA, and visualized using the streptavidin−horseradish peroxidase conjugate and a chemiluminescence method (ECL detection system). (B) Samples of a pol δ preparation at the crude extract, phenyl−Sepharose, immunoaffinity, ssDNA cellulose, and Superose 12 gel filtration steps were subjected to SDS−PAGE and analyzed using biotinylated PCNA as described above. Recombinant p50 (2.5 μg) was also run on this gel, showing that it is not overlayed by PCNA. The positions of the prestained protein standards are marked on the sides. The positions of the p125 and p68 bands are shown by the arrowheads.

nondenaturing gel electrophoresis in gradient gels under conditions where limiting mobility of the proteins was reached. Under these conditions, the migration of proteins or stable protein complexes can be correlated with their relative molecular weights. Our analyses of calf thymus pol δ purified through the initial DE-52 column revealed a single high-molecular weight complex ($M_r \sim 520\,000$) by Western blotting against a pol δ p125 antibody (Figure 5A, lane 1). Similar results were obtained when the material was purified through a subsequent Q-Sepharose column (Figure 5A, lane 2). This monodisperse behavior of the pol δ complex on native gel electrophoresis is striking and argues for the maintenance of a very discrete complex under these conditions. Examination of the purified heterodimeric form of pol δ under the same conditions gave a single band at 175 000 (not shown). Further analysis was performed by excision of the 520 kDa bands obtained from nondenaturing gel electrophoresis. These were subjected to SDS−PAGE, and assessed by PCNA overlay analysis. The results revealed the presence of polypeptide bands at 125 and 68 kDa, and a band at ca. 58 kDa. The latter is not the small subunit of the pol δ (p50) which does not interact with PCNA in the overlay analysis (*21*). This work further confirmed that p68 is strongly associated with high-molecular weight forms of pol δ. The results of these experiments support those shown above for the presence of a PCNA binding protein of ca. 68 kDa which is associated with the pol δ heterodimer in a high-molecular weight complex. In addition, these experiments indicate the possible existence of yet another novel PCNA binding protein of about 58 kDa that is associated with the complex, although we cannot rule out the possibility that it is a proteolytic product of the 125 kDa catalytic subunit.

Sensitivity to PCNA. When the response of the immunoaffinity- and gel filtration-purified enzyme to PCNA was compared to that of the heterodimer, it was observed that the former consistently exhibited a higher response than the latter. To eliminate the possibility that this was due to

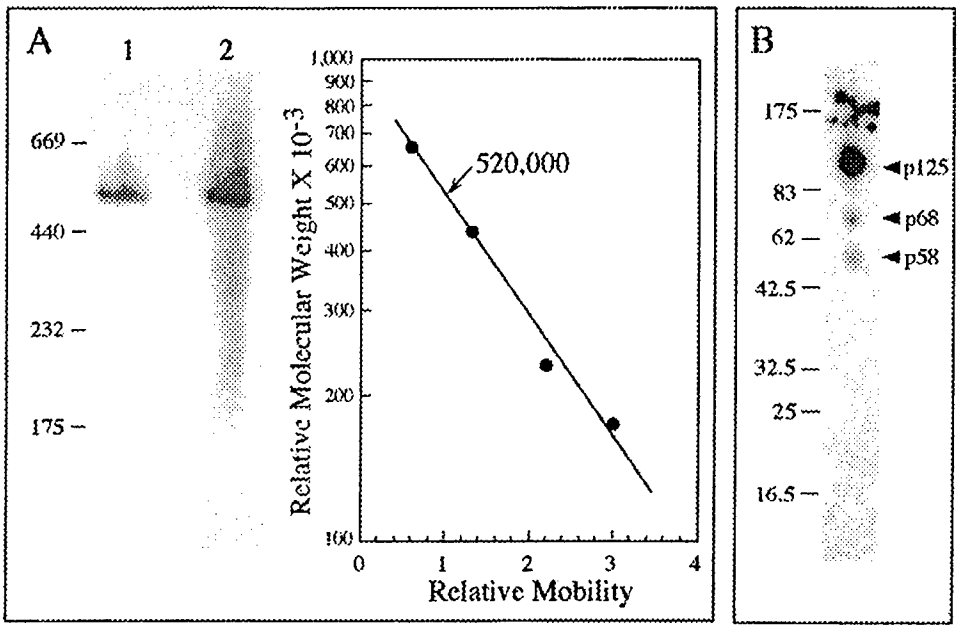

FIGURE 5: Native gel electrophoresis of pol δ. (A) Nondenaturing gel electrophoresis of pol δ. Calf thymus pol δ that was purified through the DE-52 step (lane 1) and then further purified on a Q-Sepharose column (lane 2) was subjected to electrophoresis on a gradient gel (5 to 15% acrylamide) under nondenaturing conditions and electrophoresed until the marker proteins had reached a limiting mobility (Experimental Procedures). The proteins were then transferred to nitrocellulose membranes which were then Western blotted with an antibody against the p125 subunit of pol δ. The diagram on the right shows the estimation of the size of the pol δ complex. Protein standards were used to estimate the size of the complex containing the p125 subunit of pol δ. These were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The arrow shows the migration position and estimated size of the protein complex containing the pol δ p125 subunit. (B) Two-dimensional PCNA overlay analysis. The gel slice from the first dimension corresponding to the 520 kDa band was cut with reference to the Western blot, immersed in SDS−PAGE sample buffer without shaking for 2 h at 37 °C, and then secured on top of the stacking gel of a 5−15% SDS−polyacrylamide gradient gel with 0.5% agarose in SDS sample buffer without glycerol. The proteins were transferred onto a nitrocellulose membrane, and the PCNA overlay was performed as described in Figure 4. The positions of the p125, p68, and p58 bands are marked by arrows.

differences in assay conditions, a systematic comparison was made of the PCNA responses of (a) the recombinant human p125 catalytic subunit, (b) a p125 mutant in which the N-terminus containing the PCNA binding region was deleted (*10*), (c) the recombinant heterodimer produced by coexpression in Sf9 cells, (d) the calf thymus heterodimer isolated as described previously by immunoaffinity and ssDNA cellulose chromatography (*14*), and (e) the immunoaffinity-purified calf thymus pol δ preparation. Different time points and concentrations of PCNA were used to optimize the assay conditions, and the same preparation of human PCNA was used for all the assays. The results (Figure 6) show that the p125 catalytic subunit has a small but detectable response to PCNA, which is eliminated by deletion of the N-terminus. The recombinant human p125/p50 heterodimer and the calf thymus heterodimer isolated to near homogeneity were activated by PCNA, to comparable extents, about 12−16-fold, in contrast to the immunoaffinity-purified enzyme, which was activated by nearly 40-fold. This suggested that components required for the full response to PCNA were removed from the immunoaffinity-purified pol δ during its purification to the heterodimer.

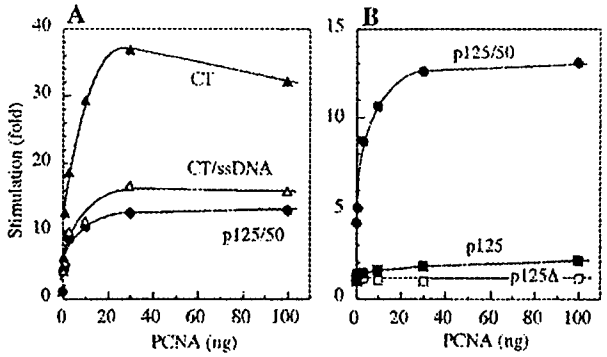

FIGURE 6: PCNA sensitivity of pol δ after immunoaffinity and ssDNA cellulose chromatography. Pol δ activity was determined using poly(dA)/oligo(dT) as the template−primer complex, and results are shown as the fold stimulation of activity in the presence of varying amounts (nanograms) of PCNA per 50 μL assay. Linearities of the assays were determined by time course measurements (not shown). (A) The black triangles show the response of immunoaffinity-purified calf thymus pol δ (CT). The white triangles show the same preparation after subsequent purification to a homogeneous heterodimer on ssDNA cellulose and heparin−agarose chromatography. The black circles show the PCNA response of recombinant human pol δ p125/p50 obtained by coexpression of the two subunits in Sf9 cells. (B) The response of the recombinant human pol δ p125/p50 heterodimer is compared with that of the recombinant p125 subunit (■) and its deletion mutant (p125Δ) in which the 186 N-terminal amino acids were deleted (□). All recombinant human pol δ proteins were expressed in Sf9 cells and purified as previously described (*40*). In these assays, the same preparation of purified calf thymus PCNA was used rather than recombinant PCNA, and all assays were conducted in the same experiment.

DISCUSSION

DNA polymerase δ is now well recognized as the key DNA polymerase in eukaryotic DNA replication, and the p125 and p50 subunits are well conserved between mammals and yeast (*15*, *27*). There are questions as to the complete polypeptide composition of pol δ, as well as that of other

associated replication proteins that together with pol δ form the multiprotein assembly that functions in chromosomal replication. In addition, evidence which shows that pol δ is also involved in DNA repair processes (*28*, *29*) indicates that the pol δ holoenzyme may associate dynamically with different proteins to form more than one type of higher-order assembly. As noted in the introductory section, extensive investigation in prokaryotes of *E. coli* DNA polymerase III has led to the identification of a number of polypeptides which have been studied in various subassemblies. The investigation of the identity and functions of replication proteins in mammalian systems is difficult because of the small amounts of material available, and because these protein complexes are likely to be dissociated upon most conventional methods of isolation, including gel filtration. In this study, we have taken advantage of a method for the immunoaffinity purification of pol δ from calf thymus, which yields relatively large amounts of enzyme, sufficient for both a proteomics approach and the possibility that the methods might be gentle enough to allow isolation of total or partial assemblies of the pol δ heterodimer associated with its natural protein partners.

Investigation of the behavior of the immunoaffinity-purified pol δ revealed that it can be shown to exist in protein complexes that are much larger (>250000) than the heterodimer. Moreover, these complexes represent assemblies that are highly purified and contain the heterodimer as the primary polypeptide component. Our studies indicate that the immunoaffinity purification does in fact lead to isolation of the heterodimer with at least some of its associated proteins. For the preliminary purification steps, we have used batchwise, rather than gradient elution, methods to minimize separation of associated proteins, and used the DEAE-cellulose and phenyl−Sepharose supports originally devised for the purification of pol δ (*13*). We have also found that ssDNA cellulose, as well as heparin−agarose chromatography, which are efficient for the isolation of the heterodimer, may do so by removing other associated proteins.

Comparison of the gel filtration behavior of the heterodimeric and the immunoaffinity-purified pol δ revealed a shift in the native molecular weight of 175 000 of the pol δ core to more than 500 000. As observed by Maki et al. (*4*), gel filtration of diluted pol III* leads to its dissociation from 800 kDa to fractions corresponding to 530, 480, and 380 kDa in size. The studies reported here provide the first evidence for high-molecular weight forms of mammalian pol δ in a highly purified state.

In other studies, we have found a number of replication proteins bound to PCNA Sepharose (*20*). As with other highly complex protein assemblies, e.g., *E. coli* DNA polymerase or RNA polymerase, conventional methods of isolation seldom yield complete stoichiometric complexes. Examination of the polypeptide compositions of the immunoaffinity-purified material revealed that while the p125 and p50 polypeptides were the major constituents, the preparation contained a number of other polypeptides which remain associated with the heterodimer during gel filtration or glycerol gradient ultracentrifugation. Clearly, some of these may represent impurities, while others may represent bona fide components of a mammalian replication complex. In this study, microsequencing was used to identify some of the associated polypeptides. The significant findings are the identification of KIAA0039 and its proteolytically derived peptides, as well as the 70 and 32 kDa subunits of RPA, as components that are associated with the high-molecular weight form of pol δ. During the course of this study, Hughes et al. (*30*) also identified KIAA0039 as a PCNA binding protein by using PCNA affinity chromatography, and have proposed that this is the third subunit of mammalian DNA polymerase δ, on the basis of the similarity of the sequence with that of Cdc27, and the association of the protein with pol δ on glycerol gradient ultracentrifugation. In their studies, they noted that recombinant KIAA0039 protein exhibited an anomalous migration on SDS−PAGE as a protein of 66 kDa, while its calculated molecular mass is 51.4 kDa. This is consistent with our observations on the behavior of the protein. Two reasons were put forth by Hughes et al. (*30*) to explain the previous failure to detect p66 in purified pol δ preparations. One was that this subunit is refractory to certain silver-staining reagents, and the other was that p66 is not absolutely required for polymerase activity and may have been lost during the lengthy purification procedures. The studies presented here provide stronger evidence that the 68 kDa polypeptide is a likely subunit of pol δ. The presence of the proteolytic products of p68 indicates that the protein is susceptible to nicking, and provides a reasonable explanation for the variability of its appearance in highly purified pol δ preparations. In previous work in this laboratory, p68 was found to be a persistent "impurity" that is associated with the immunoaffinity-purified enzyme and is only removed by a combination of ssDNA cellulose and heparin−agarose chromatography (*14*). The native gel electrophoresis experiments described in this work provide additional evidence for the association of p68 with pol δ. Pairwise alignments using the Clustal W 1.8 program show that there is only 15−16% of sequence identity between Pol32p and Cdc27, Pol32p and p68, and Cdc27 and p68. However, evaluation of the significance of the alignment score for p68 and Cdc27 using the PRSS program (http://www.expasy.ch/tools) provided a score of 0.4; i.e., the alignment score would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 time in 100 attempts. This indicates that the degree of similarity between these two proteins is significant. Interestingly, all three sequences possess a PCNA binding motif at their C-termini. The finding that p68 is a PCNA binding protein may be responsible for the observations that the immunoaffinity-purified enzyme shows a greater PCNA response than the p125/p50 heterodimer.

If p68 were to associate with the pol δ heterodimer in a stoichiometric fashion, the expected molecular mass of the complex would be 226 kDa; given that the KIAA0039 protein behaves on SDS−PAGE with an anomalous molecular mass of 68 kDa, an upper limit for a relative molecular weight for the complex of 243 000 can be projected. This is at the lower limit of the size range (from 250000 to >500000) that was found for the behavior of the immunoaffinity-purified enzyme on gel filtration, and much smaller than the size of pol δ found on nondenaturing gel electrophoresis (ca. 520 000). The explanations for this could be that there are additional subunits or associated proteins in the complex, or that the trimeric species is capable of dimerization. The current experimental information cannot distinguish between these possibilities. However, it is relevant

that in *S. cerevisiae*, the cognate third subunit encoded by POL32 has been shown to be able to form a hexameric protein with the two classical subunits of pol δ, so that it has been suggested to be a dimerization factor (*17*, *18*). This is not inconsistent with the behavior of pol δ that was observed here, since a hexameric complex would have a molecular weight of about 490 000, but more rigorous studies using reconstituted subunits will be needed to establish this. Gerik et al. (*17*) reported no other subunits in the most purified preparation of pol δ in the yeast *S. cerevisiae* besides p125, p58, and p55. In *S. pombe*, one additional polypeptide was identified as a potential subunit of pol δ that is encoded by the Cdm1 gene (*16*).

The issue of whether there is a subunit of eukaryotic pol δ that confers the property of dimerization is of some significance, since there is evidence that pol δ functions in both the leading and lagging strand synthesis (*6*, *29*). This would suggest that the mechanism for concerted DNA synthesis that depends on a dimerization factor that couples two DNA polymerase enzymes as in the *E. coli* system is conserved in eukaryotic systems (*6*). In *E. coli*, the tau protein serves to maintain a dimeric DNA polymerase, while the *S. cerevisiae* heterotrimer has been shown to dimerize, with the implication that the third subunit may serve a parallel function (*2*, *29*). At the present time, we cannot eliminate the possibility that the larger forms of pol δ activity that we observe may be due to the presence of a dimerization factor.

The second significant finding that was made was the presence of two of the three subunits of RPA in the immunoaffinity-purified pol δ preparations. The finding that RPA, or HSSB (human single-stranded DNA binding protein), is also present in the high-molecular weight form of pol δ after FPLC gel filtration suggests that there may be an interaction of RPA with the replication complex. RPA is an abundant multimeric protein consisting of three subunits (p70, p34, and p14) which is essential for DNA replication and is also involved in DNA repair and recombination (*31*). It binds tightly to single-stranded DNA and affects the activity of other replication proteins, e.g., T antigen, DNA polymerases α (*32*) and δ (*33*), p53 (*34*), and transcriptional initiators, e.g., GAL4 and VP16 (*35*). These findings are consistent with previous studies in which the presence of RPA could be detected by Western blotting of pol δ preparations isolated by PCNA affinity chromatography (*20*). While the presence of RPA in highly purified pol δ complexes may be fortuitous, there are a number of studies which indicate the likelihood that RPA itself may interact with the DNA polymerases in the replication complex. Mutation of the zinc finger domain of RPA has been shown to eliminate DNA replication activity (*36*). Genetic evidence in yeast indicates that the p70 subunit interacts with both pol α and pol δ (*37*). In the complete SV40 DNA replication system, neither prokaryotic, yeast, nor viral SSBs (single-stranded DNA binding proteins) can replace human RPA, thus suggesting that it may participate in specific protein−protein interactions with other replication proteins (*38*). More recently, Yuzhakov et al. (*39*) demonstrated a direct interaction between the pol δ heterodimer and RPA. The p70 subunit of RPA bound to the pol δ heterodimer, but not the p34−p14 subcomplex of RPA. RFC, the clamp loader for PCNA, also binds to pol δ. The binding of pol δ and RPA was found to compete for binding to RFC. In studies of the interactions of these proteins, Yukazhov et al. (*39*) have proposed that RPA forms an important touchpoint for the assembly of the pol δ replication complex. Our findings that RPA is present in a purified high-molecular weight complex of pol δ provide further evidence that the interaction of RPA and pol δ is a significant one.

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Identification of a Fourth Subunit of Mammalian DNA Polymerase δ*

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A 12-kDa and two 25-kDa polypeptides were isolated with highly purified calf thymus DNA polymerase δ by conventional chromatography. A 16-mer peptide sequence was obtained from the 12-kDa polypeptide which matched a new open reading frame from a human EST (AA402118) encoding a hypothetical protein of unknown function. The protein was designated as p12. Human EST AA402118 was identified as the putative human homologue of *Schizosaccharomyces pombe* Cdm1 by a tBlastn search of the EST data base using *S. pombe* Cdm1. The open reading frame of human EST AA402118 encoded a polypeptide of 107 amino acids with a predicted molecular mass of 12.4 kDa, consistent with the experimental findings. p12 is 25% identical to *S pombe* Cdm1. Both of the 25-kDa polypeptide sequences matched the hypothetical KIAA0039 protein sequence, recently identified as the third subunit of pol δ. Western blotting of immunoaffinity purified calf thymus pol δ revealed the presence of p125, p50, p68 (the KIAA0039 product), and p12. With the identification of p12 mammalian pol δ can now be shown to consist of four subunits. These studies pave the way for more detailed analysis of the possible functions of the mammalian subunits of pol δ.

DNA polymerase δ (pol δ) is the key polymerase that is involved in the replication of chromosomal DNA in eukaryotic cells. Studies of the *in vitro* replication of SV40 DNA have established that pol δ plays a central role in mammalian DNA replication (1). Proliferating cell nuclear antigen (PCNA),[1] the molecular sliding clamp of pol δ, is a processivity factor for pol δ and ε (2). PCNA was first identified as an activating factor for pol δ (3, 4) and is essential for replicative DNA synthesis. Several other factors have been identified as being required for SV40 DNA replication. Replication factor C (also known as activator-1) binds to the primer-template terminus, following which it recruits PCNA and then pol δ (5, 6) onto the DNA template. Replication Protein A, the single stranded DNA-binding protein, is involved in both initiation and elongation and also stimulates pol δ activity when replication factor C and PCNA are present (7, 8). The current view of DNA replication at the replication fork is that the pol δ complex is responsible for synthesis of the leading strand and that pol δ also participates in synthesis of the lagging strand (1). DNA polymerase α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, and the actual elongation of the primers is performed by DNA polymerase δ in a process that requires "polymerase switching" (9). Additional proteins, including topoisomerase and helicase activities, are also involved in the movement of the replication fork (1).

Mammalian pol δ has been rigorously isolated by conventional methods as a heterodimer consisting of two subunits, p125 and p50 (3). The subunit structure of pol δ has been the focus of recent investigations in yeast, and these have led to the identification of additional subunits. In *Schizosaccharomyces pombe*, pol δ is believed to consist of at least four subunits: a large catalytic subunit (Pol3) and three smaller subunits (Cdc1, Cdc27, and Cdm1) (10, 11). Pol δ purified from *Saccharomyces cerevisiae* is composed of three subunits: Pol3p, Pol31p/Hys2, and Pol32p (12–14). The pol δ core purified from calf thymus consists of two subunits: p125 and p50 (3). However, we have found that recombinant p125 catalytic subunit alone can only be stimulated by PCNA by 2-fold at most, while the overexpressed p125/p50 heterodimer is stimulated much less than pol δ purified by immunoaffinity chromatography (15, 16). These findings suggest that additional factor(s) which may be removed during protein purification are required for a full PCNA response in our assay. This is consistent with the hypothesis that mammalian pol δ may also contain additional subunits.

Using the proteomics approach, by peptide sequencing of polypeptides associated with the core pol δ in highly purified preparations isolated by p125 immunoaffinity chromatography, we have previously identified a 68-kDa polypeptide that is encoded by KIAA0039 and which is associated with the pol δ core. The p68 polypeptide is the third subunit of mammalian pol δ (17). Using a combination of proteomic approaches and GenBank searches, we have identified a novel subunit of pol δ that is the mammalian homologue of Cdm1, which in *S. pombe* is the fourth subunit of pol δ. Mammalian pol δ may thus consist of at least four subunits.

EXPERIMENTAL PROCEDURES

Materials—cDNA AA402118 was obtained from ATCC (Rockville, MD). Calf thymus tissue was obtained from Animal Technologies (Tyler, TX). Q-Sepharose, SP-Sepharose, heparin-Sepharose, Mono Q columns, and Mono S columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Purification of Calf Thymus Pol δ—The immunoaffinity purification was performed as described previously by Jiang *et al.* (18).

Conventional Purification of Calf Thymus Pol δ—The following buffers were used: lysis buffer consisted of 50 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, 10 mM benzamidine. TGEED buffer consisted of 50 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; pol, polymerase; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

TABLE I
Purification of calf thymus DNA polymerase δ

Fraction	Total volume	Protein	Total units	Specific activity
	ml	*mg*		*units/mg protein*
DE 52	3,615	18,075	397,000	22
Q-Sepharose	470	2,585	295,000	113
SP-Sepharose	176	722	468,000	648
Mono Q	34	76.5	56,000	736
Heparin-Sepharose	17	12.2	39,000	3,211
Mono S	3.3	2.1	9,400	4,500
Source Q15	3.0	0.15	900	6,000
Superdex 200	0.8	0.022	200	8,900

5% glycerol. KGEED buffer consisted of 20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.

All steps were carried out at 0–4 °C. Pol δ activity was assayed using poly(dA)/oligo(dT) as a template (19). Eight hundred grams of frozen calf thymus tissue in 4 liters of lysis buffer were homogenized in a Waring blender. The suspension was centrifuged at 5,000 rpm at 4 °C for 1 h and filtered through glass wool. The supernatant was mixed with 1.5 liters of DE52-cellulose equilibrated with TGEED buffer and stirred for 30 min. The mixture then was filtered through a Buchner funnel. The DE52-cellulose was washed with TGEED and the pol δ activity was stripped off with 20% ammonium sulfate in TGEED buffer.

The 3.5 liters of DE52-cellulose fraction were precipitated by the addition of 320 g/liter of ammonium sulfate. The suspension was stirred for 30 min, kept on ice for an additional 30 min, and then centrifuged at 10,000 × *g* for 45 min. The precipitate was resuspended in TGEED and dialyzed against TGEED buffer containing 50 mM NaCl with two changes and applied on to a 70-ml Q-Sepharose column. The bound proteins were eluted with a linear gradient of 50–750 mM NaCl in TGEED. The peak fractions containing pol δ activity were pooled and dialyzed against KGEED buffer containing 25 mM KCl. The Q-Sepharose fraction was loaded on to a 50-ml SP-Sepharose column. Pol δ was eluted with a linear gradient of 25–650 mM KCl in KGEED. The fractions containing enzyme activity were pooled and applied to a 10-ml Mono Q column, which was equilibrated with TGEED buffer containing 25 mM NaCl. The column was washed with 40 ml of TGEED buffer containing 25 mM NaCl. The activity was eluted with a gradient of 25–650 mM NaCl in 100 ml of TGEED at a flow rate 0.4 ml/min. The Mono Q fractions were pooled and dialyzed against TGEED buffer containing 25 mM NaCl and applied to a 5-ml heparin-Sepharose column. The column was washed with 2 column volume of TGEED containing 25 mM NaCl and eluted with a 50-ml gradient of 25–750 mM NaCl in TGEED at a flow rate 0.5 ml/min.

The heparin-Sepharose fraction (17 ml) was dialyzed against two changes of KGEED buffer containing 50 mM KCl and loaded onto a 1-ml Mono S column equilibrated with KGEED buffer. The column was washed with 5 ml of KGEED buffer and then eluted with a 20-ml linear gradient of KGEED buffer from 50 to 700 mM KCl. The active fractions were combined and dialyzed against TGEED buffer until the conductivity reached that of TGEED containing 50 mM NaCl. The fraction was applied to a Source Q15 column. The enzyme was eluted with a linear gradient of 50–650 mM NaCl in TGEED. The fractions with enzyme activity were pooled (3.0 ml) and concentrated to 270 μl using Centricon 30 (30,000 MW cutoff, Amicon). The concentrated enzyme (270 μl) was chromatographed on a FPLC Superdex 200 column equilibrated with TGEED buffer containing 150 mM NaCl. Fractions above 50% of the maximum peak of activity were pooled.

Protein Sequence Analysis—Polypeptide bands excised from a Coomassie Blue-stained gel were used for protein sequence analysis by the Harvard Microchemistry Facility using a microcapillary reverse-phase high performance liquid chromatography nano-electrospray tandem mass spectrometry (μLC-MS-MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

Antibodies—Peptide rabbit polyclonal antibodies against p12/hCdm1 and p68 were generated from a commercial source (SynPep, Dublin, CA) and purified by a peptide affinity column made from the same peptide antigen. For p12, the peptide contains amino acid residues 77 to 94 of p12 (H_2N-GLEPPPEVWQVLKYHPGD-COOH). For p68 (encoded by KIAA0039) the 19-amino acid peptide from near the extreme N terminus of p68 was used (H_2N-TDQNKIVTYKW-LSYTLGVH-COOH).

Western Blot Analysis—Proteins were transferred to 0.45 μM nitrocellulose membranes (Bio-Rad) after SDS-PAGE in transfer buffer (25 mM Tris-HCl, 192 mM glycine containing 10% v/v methanol) in a Genie blotter (Idea Scientific, Minneapolis, MN) for 75 min for 0.8-mm thick gels using a constant voltage of 12 volts. The membrane was incubated in TBST buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% Tween 20) containing 5% fat-free dry milk for 1 h at room temperature and washed briefly with TBST. The membrane was incubated with primary antibody for 1 h at room temperature or overnight at 4 °C. The membrane was washed 3× with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Pierce, Rockford, IL) for 1 h. The membrane was washed 3× with TBST. SuperSignal West Pico Chemiluminescent Substrate was used for signal production (Pierce) and the signal was captured on a Blue Bio film (Denville Scientific, Metuchen, NJ) after exposure for 15 s to 30 min and developed.

RESULTS

Demonstration That p68 and p12 Are Subunits of Mammalian Pol δ—We have previously devised a conventional procedure for the rigorous isolation of the pol δ core enzyme containing p125 and p50 (3). In order to isolate a multisubunit form of mammalian pol δ, a new purification scheme was devised, which allowed the isolation of pol δ core that retained associated polypeptides. This involved successive chromatographies on DE52, Q-Sepharose, SP-Sepharose, Mono Q, heparin-Sepharose, Mono S, Source Q15, Superdex 200 supports, including four FPLC chromatography steps (Mono Q, Mono S, Source Q15, and Superdex 200). Table I shows the purification of pol δ by this means. The specific activity of the preparation (about 9,000 units/mg) was comparable with that of pol δ purified by immunoaffinity chromatography (18). Review of a number of preparations isolated by the latter method gave an average specific activity about 10,000 units/mg, with a PCNA stimulation of 20–40-fold. The PCNA stimulation of 30-fold was found for the preparation obtained by the new procedure. This is similar to that of the immunoaffinity purified enzyme. The average specific activity of the purified recombinant pol δ heterodimer in our hands is about 2000 units/mg, with maximum PCNA stimulations of 6–10-fold. These results indicate that rigorously purified pol δ p125/p50 heterodimer has lost a significant fraction of its ability to respond to PCNA.

The final purification step used was FPLC gel filtration on Superdex 200. Calibration of the column showed that the peak of pol δ activity was eluted at a position indicating a much higher molecular weight (280,000) than can be accounted for by the two-subunit core. The Coomassie Blue staining of this pol δ complex is shown in Fig. 1. There were six major bands in the peak fractions of pol δ from fractions 48 to 50; these were of 125 kDa, 50 kDa, a doublet at about 25 kDa and a doublet at about 12 kDa. These polypeptide bands were excised from the Coomassie Blue-stained gel and sequenced at the Harvard Microchemistry Facility using LC/MS/MS methods. The sequencing results are displayed in Table II. Both the 25-kDa polypeptides were identified as the proteolytic products of KIAA0039, which was recently found to be associated with pol δ by a PCNA overlay assay (17). The KIAA0039 product was also eluted with pol δ from a PCNA affinity column (20). Our data support the view that p68 is the mammalian third subunit of pol δ but

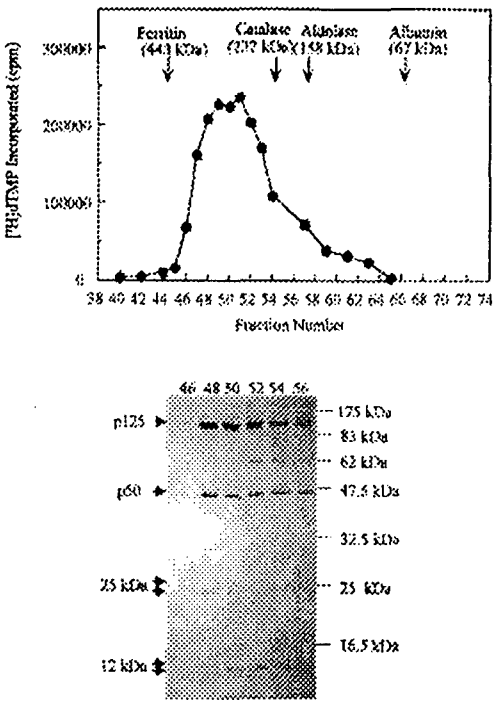

FIG. 1. **Chromatography of the purified calf thymus pol δ complex on Superdex 200.** The figure shows the elution profile of the calf thymus pol δ at the final purification step ("Experimental Procedures"). *Upper panel*, the activity of pol δ in the fractions of Superdex 200 gel filtration chromatography was assayed using poly(dA)/oligo(dT) as the template in the presence of PCNA. *Lower panel*, the peak fractions from the Superdex 200 column were separated on a 10% SDS-polyacrylamide gel and stained for protein with Coomassie Blue. 25-kDa upper and lower and 12-kDa upper and lower, as well as the p125 and p50 pol δ core subunits are marked by *arrows*.

TABLE II
Peptide sequences data from calf thymus pol δ complex

Identity	Peptide mass on SDS-PAGE	Peptide sequences obtained
Pol δ p125	125 kDa	IFEPILGEGR DPGSAPSLGDRVPYVIISAAK
Pol δ p50	50 kDa	FLGTSGQNVSDIFR AMPLQPSILR TQAASVEAVK
Pol δ p68[a] (KIAA0039)	25-kDa upper	VAVVREDKLEAVK QMLYDYVER
Pol δ p68[a] (KIAA0039)	25-kDa lower	WLSYTLGVHVNQAK FSAIQCAAAVPR DSGPLFNTDYDILK GIMGMFASK QMLYDYVER
Keratin 16	12-kDa upper	ADLEMQIENLR
Pol δ p12	12-kDa lower	QFDLAWQYGPCTGITR

[a] The protein has a size of 68 kDa present in p125-immunoaffinity purified calf thymus pol δ. In this purification scheme, it was proteolysed into two 25-kDa species.

indicate that it is highly susceptible to proteolysis.

The sequence of the upper 12-kDa band showed that this was derived from keratin. The second 12-kDa polypeptide was found to be a novel protein. The partial sequence obtained from this protein was QFDLAWQYGPCTGITR (Table II). This sequence was searched against the known protein data bases, which did not provide a match. A tBlastn search of the EST data base showed a match with one human EST sequence, AA402118, which, however, did not have a well defined open reading frame. Concurrently, the *S. pombe* Cdm1 protein sequence, which represents the fourth subunit of pol δ, was used for a tBlastn search at NCBI. This also retrieved the human EST sequence, AA402118. The EST cDNA clone was obtained from ATCC and was resequenced and corrected. The corrected sequence contained an open reading frame that encoded a protein of 107 amino acid residues, with a predicted molecular mass of 12.4 kDa. This protein was designated as p12. The corrected DNA sequence has been deposited in GenBank with the accession number AJ179890 (Fig. 2). The peptide sequence obtained from p12 shows a perfect match with residues 51–65 of the open reading frame of AJ179890.

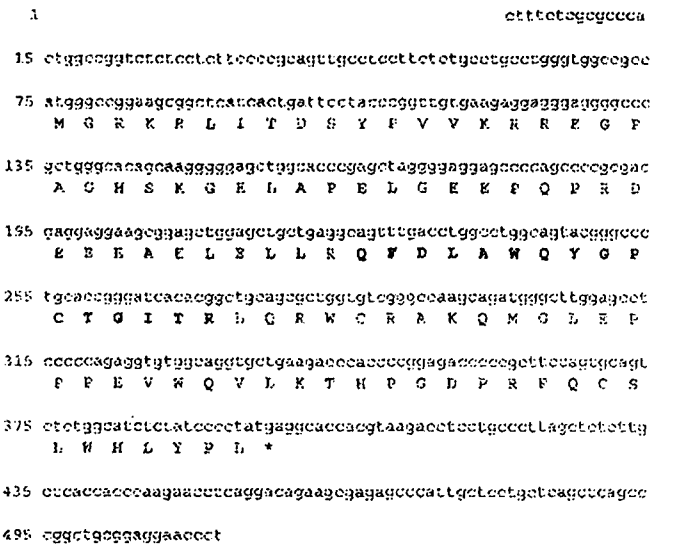

FIG. 2. **Nucleotide sequence and predicted amino acid sequence of the smallest subunit of mammalian DNA polymerase δ.** This figure shows the nucleotide sequence of the human EST AA402118 which was corrected by resequencing (AJ179890). The insert of the human EST A402118 in the cloning vector pT7T3D is 512 base pairs, and encodes a protein of 107 amino acids. The amino acid sequence of the peptide derived from the 12-kDa polypeptide in the calf thymus pol δ preparation (Fig. 1) is shown in ***bold***.

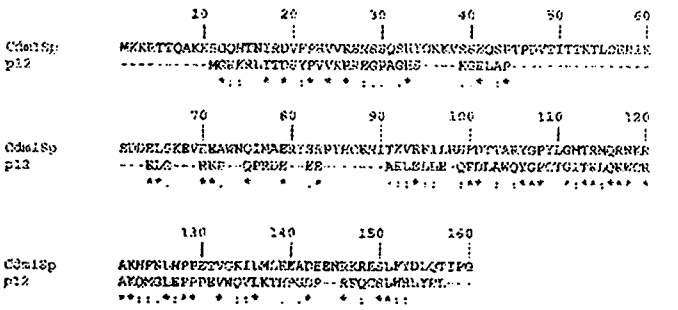

FIG. 3. **Alignments of human p12 with *S. pombe* Cdm1.** Human p12 (AJ179890) and *S. pombe* Cdm1 (emb AJ006032) were aligned with the Clustal 1.74 program. The identity between these two proteins is 25% and the similarity is 39%.

These results indicate that p12 is a likely human homologue of the *S. pombe* Cdm1 protein, which has been reported to be the fourth subunit of *S. pombe* pol δ (10, 21). The *S. pombe* Cdm1 protein has a calculated molecular mass of 18.5 kDa and an apparent size of 22 kDa on SDS-PAGE, and is significantly larger than human p12. Sequence alignments were performed to assess the possible relationships between these two proteins. Protein sequence alignment indicates that the identity between p12 (107 residues) and *S. pombe* Cdm1 (160 residues) is 25% and the similarity is 39% (Fig. 3). It can be seen that the main region of identity of p12 is with the C-terminal half of *S. pombe* Cdm1. Alignment of amino acid residues 96- 142 of Cdm1 with

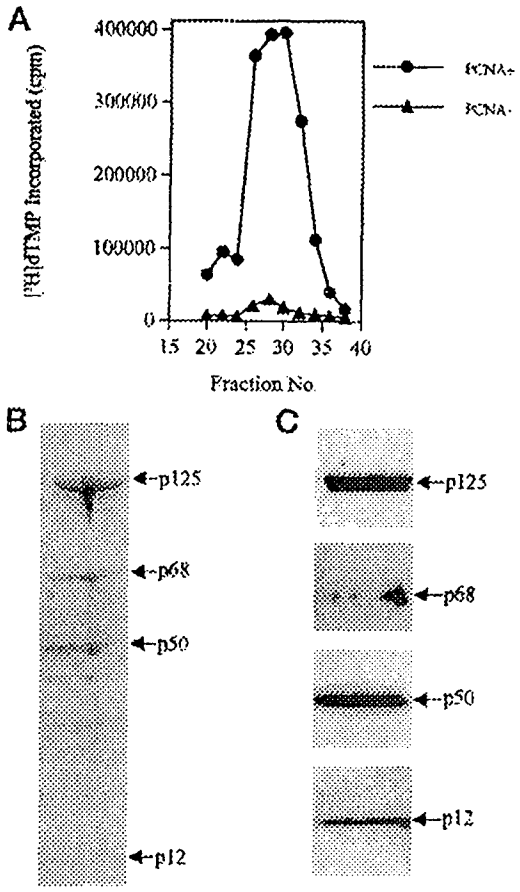

FIG. 4. **Western blot analysis of pol δ subunits purified by p125-immunoaffinity chromotography from calf thymus.** Calf thymus pol δ was purified through DE52, phenyl-agarose, and p125 immunoaffinity column chromatographies in the presence of protease inhibitors (18). *Panel A,* activity assay of the fractions eluted from the immunoaffinity column using poly(dA)/oligo(dT) as the template in the absence (*closed triangles*) and presence (*closed circles*) of PCNA. *Panel B,* Coomassie Blue staining of peak fraction number 28. *Panel C,* Western blotting of peak fraction number 28 using monoclonal antibody 78F5 against p125, 13D5 against p50, and rabbit peptide polyclonal antibodies against p68 and p12.

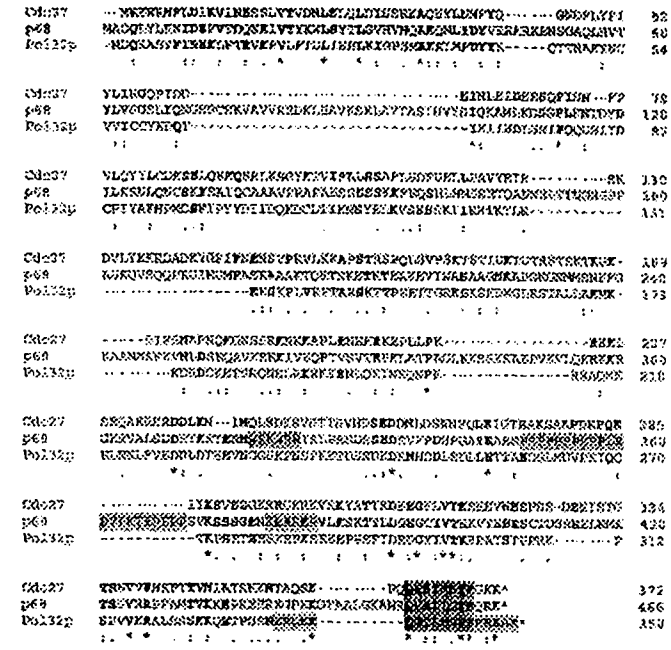

FIG. 5. **Multiple sequence alignment of p68, Pol32p, and Cdc27.** p68 (the protein product of KIAA0039, BAA05039) with *S. pombe* Cdc27 (P30261) and *S. cerevisiae* Pol32p (CAA89571) were analyzed using the Clustal 1.8 program. The PCNA binding motif is highlighted in *dark gray*. The putative nuclear localization signals of p68 and Pol32p are highlighted in *light gray*. The unique proline-rich motif in p68 is also highlighted.

TABLE III
Summary of pol δ subunits

DNA polymerase δ	Mammalian	*S. pombe*[a]	*S. cerevisiae*[b]
The catalytic subunit	p125	125 kDa, Pol3	125 kDa, Pol3p
The second subunit	p50	55 kDa, Cdc1	58 kDa Pol31p
The third subunit	p68[c]	54 kDa, Cdc27	55 kDa Pol32p
The fourth subunit	p12[d]	22 kDa, Cdm1	Not found

[a] Isolated from *S. pombe* (10).
[b] Reconstituted as a three-subunit enzyme (13).
[c] Identified as bovine p68 (encoded by KIAA0039) in p125 immunoaffinity purified pol δ (17). Isolated by PCNA affinity chromatography of mouse FM3A cell extracts (20).
[d] Identified in this study.

residues 48 to 94 of p12 shows that there is a 44% identity. This degree of similarity is sufficient for p12 to be regarded as the mammalian homologue of *S. pombe* Cdm1. Taken together with the sequence identification of the p12 and its co-purification with the calf thymus pol δ core through eight chromatography procedures, these findings provide strong evidence for the identification of p12 as a novel subunit of mammalian pol δ.

Western Blot Analysis of Immunoaffinity Purified Pol δ—We had previously shown that pol δ isolated by immunoaffinity chromatography contains the pol δ core in association with a number of other polypeptides (18), and also displayed a much higher molecular weight than could be accounted for by the core on gel filtration analysis (22). The failure to observe p12 in these studies could be due its small size and the fact that it migrated close to the dye front under the conditions used. A preparation of pol δ was purified from calf thymus using immunoaffinity chromatography (18) and the preparation was assessed for the presence both of the p68 and p12 subunits. The presence of these two polypeptides on SDS-PAGE gels of the preparation are shown in Fig. 4. Polypeptides corresponding to 68 and 12 kDa were prominent components of the preparation, and their identity as the p68 and p12 polypeptides was confirmed by Western blotting (Fig. 4). Thus, the presence of all four subunits of pol δ (p125, p50, p68, and p12) were demonstrated in this preparation (Fig. 4). The KIAA0039 product in the Western blot was 68 kDa.

p68 Is the Third Subunit of Mammalian Pol δ, the Homologue of S. pombe Cdc27 and S. cerevisiae Pol32p—The p68 sequence has a conserved $p21^{Waf1}$-like PCNA binding motif at the extreme C terminus, as does *S. pombe* Cdc27 and *S. cerevisiae* Pol32p, the yeast third subunits of pol δ (13). The p68 sequence encoded by KIAA0039 was aligned with the sequences of Cdc27 and Pol32p (Fig. 5). Analysis of the alignments showed that p68 shares little sequence identity with Cdc27 and Pol32p. The only sequence conservation was the C-terminal PCNA binding motif in these three sequences. p68 and Pol32p both have nuclear localization motifs. p68 also has an unique proline-rich motif. Pairwise alignments using the Clustal W 1.8 program show that between Pol32p and Cdc27, Pol32p and p68, or Cdc27 and p68 there is only 15 to 16% sequence identity (not shown). However, evaluation of the significance of the alignment score for p68 with Cdc27 using the PRSS program provided a score of 0.4, *i.e.* the alignment score (% identity) would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 times in 100 attempts. This indicates that the similarity between these two proteins is significant.

TABLE IV
The third subunits and putative third subunits of pol δ

Organism[a]	Accession No.	Protein	Length	PCNA binding motif
1	BAA05039	p68	466 amino acids	^{396}QVSITGFFQRK^{466}
2	CAA98958.1		445 amino acids	^{435}NSMITSFFKT^{445}
3	AAD30571.1		509 amino acids	^{499}QGNIMSFFKKV^{509}
4	AAD38629		431 amino acids	^{420}QAGIMNFFSKK^{431}
5	P30261	Cdc27	372 amino acids	^{362}QKSIMSFFGKK^{372}
6	CAA89571	Pol32p	350 amino acids	^{338}QGTLESFFKRKA^{350}

[a] 1. *Homo sapiens*; 2. *C. elegans*; 3. *A. thaliana*; 4. *D. melanogaster*; 5. *S. pombe*; 6. *S. cerevisiae*.

DISCUSSION

The thrust of the earlier studies of pol δ was the rigorous isolation of the enzyme which culminated in the isolation of a two-subunit enzyme, containing a catalytic subunit of 125 kDa and a second subunit of 50 kDa (3, 23). The major difficulties in this process were the small amounts of protein available and the likelihood of proteolysis using extensive purification schemes due to the fragile nature of the mammalian system compared with other systems, *i.e.* prokaryotic or lower eukaryotes such as yeast. Recently, expression systems for the p125 (15, 24, 25), the p50 subunit (16), and the recombinant heterodimer have been developed (26). The p50 subunit has no known enzymatic functions, but has been shown to be required for the response of the p125 subunit to PCNA (16, 26). In this study we have shown for the first time the isolation of a four-subunit mammalian pol δ enzyme. This newly isolated pol δ contains the third subunit p68 and a previously unknown subunit, p12. The latter two are the mammalian homologues of *S. pombe* Cdc27 and Cdm1, respectively.

The association of p68 and p12 with pol δ was demonstrated by their isolation with p125 and p50 from calf thymus through extensive purification involving multiple conventional column chromatographies as well as by immunoaffinity chromatography. The conventional procedure included several FPLC steps including gel permeation chromatography. The strong association of the p68 and p12 polypeptides with the pol δ core provide very strong evidence for the proposal that these represent subunits of pol δ. p68 has also been isolated from mouse cell extracts using a PCNA affinity column in association with the pol δ core consisting of the p125 and p50 subunits (20). There are extensive technical problems associated with the identification of subunits of mammalian pol δ. As encountered in our studies, these include the susceptibility of the p68 polypeptide to proteolysis and the difficulties of isolation of pol δ from animal tissues to study stoichiometries of pol δ subunits in native enzyme preparations. Nevertheless, in these studies it is demonstrated that it is possible to rigorously isolate pol δ from calf thymus in a form which retains the p68 and p12 polypeptides. A key difference in the new method from the older procedure (18) was the avoidance of single-stranded DNA cellulose chromatography.

The identification of the fourth subunit of pol δ in mammalian systems now provides a parallel for the situation found in yeast. A comparison of the subunit structures of pol δ from the mammalian and the two yeast models is shown in Table III. The catalytic subunit of mammalian pol δ is strongly conserved in evolution, and shares a high degree of homology with the corresponding catalytic subunits in *S. pombe* and *S. cerevisiae*, the identity being greater than 48% (27). The p50 subunit is less conserved than the catalytic subunit, the identity between p50 and *S. pombe* being 33% (11). Furthermore, the finding that PCNA from human or yeast origin can activate the heterologous pol δ preparations strongly suggests that the pol δ complex is functionally conserved to a high degree (28). The functions of these subunits are still incompletely understood. The third subunit of *S. pombe* pol δ was only recently identified (10) and is encoded by the *cdc27*$^+$ gene, which is needed for the transition of G_2/M in the cell cycle (11). The third subunit of *S. cerevisiae* pol δ is Pol32p, was isolated and identified in 1998. It was proposed as a candidate for dimerization factor of pol δ (13) based on the finding that the recombinant three-subunit enzyme could be shown to behave as a dimer on gel filtration (13). In addition, Pol32p was found to interact with the pol α catalytic subunit by the yeast two-hybrid method (29). These results suggest that Pol32p can (*a*) dimerize pol δ at the replication fork, and (*b*) provide a means for the proposed "polymerase" switch at the lagging strand through the interaction with pol α as suggested by Waga *et al.* (1).

p68, the mammalian homologue of *S. pombe* Cdc27, KIAA0039 was isolated from a PCNA affinity column (20) and from an immunoaffinity column of pol δ p125 (17). The third subunits of pol δ share a very low degree of similarity. In fact, Blast searches with Cdc27 failed to identify either p68 or Pol32p. tBlastn searches using Pol32p only identified a *Drosophila melanogaster* third subunit of pol δ. Similarly, using p68 the putative *Caenorhabditis elegans* and *Arabidopsis thaliana* third subunit of pol δ were identified (Table IV). As already noted ("Results"), the third subunits of human, *S. pombe,* and *S. cerevisiae* are poorly conserved, although the relationships based on the alignments can be shown to be significant. The third subunits of pol δ from different species all contain a putative p21^{waf1}-like PCNA binding motif (30, 31) at the extreme C terminus. An important aspect of the third subunit is that it interacts with PCNA, and also with the yeast p50 homologues (11, 13, 32). The ability of p68 to bind to PCNA (17, 20) may account for the loss of sensitivity to PCNA shown by pol δ p125/p50 heterodimer. In addition, all share in common a high content of charged amino acids which ranges from 29 to 35%. The calculated isoelectric points for these proteins are all basic, with the exception of the *S. pombe* Cdc27, which has an acidic isoelectric point. This common property suggests that p68 is likely to have an extended structure in solution, which is also consistent with its apparent liability to proteolysis. A third property of the third subunit may be an ability to interact with the p50 second subunit, which has been demonstrated in *S. pombe* and *S. cerevisiae* (11, 13) and also in mammalian pol δ.[2] One speculative function of p68 may be to act as a linker protein between p50 and PCNA, which would provide additional stabilization of the pol δ-PCNA interaction. This possibility is consistent with the higher sensitivity to PCNA of the pol δ preparations which contain p68 compared with that of the heterodimer.

Thus far, the fourth subunit has only been identified in mammalian sources in this present work, and previously as Cdm1 in *S. pombe*. Interestingly, data base searches have failed to identify a homologue in *S. cerevisiae*, despite the fact that its entire genome has been cloned. This may be due to a lack of evolutionary conservation. The functions of this newly

[2] L. Liu and M. Y. W. T. Lee, unpublished observations.

described subunit also remain to be determined.

In summary, this work provides evidence for the identification of a novel subunit, p12, as a component of mammalian pol δ, as well as evidence for the isolation of pol δ in a form that contains the core heterodimer in association with both p12 and the third subunit, p68.

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Identification of a Fourth Subunit of Mammalian DNA Polymerase δ*

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A 12-kDa and two 25-kDa polypeptides were isolated with highly purified calf thymus DNA polymerase δ by conventional chromatography. A 16-mer peptide sequence was obtained from the 12-kDa polypeptide which matched a new open reading frame from a human EST (AA402118) encoding a hypothetical protein of unknown function. The protein was designated as p12. Human EST AA402118 was identified as the putative human homologue of *Schizosaccharomyces pombe* Cdm1 by a tBlastn search of the EST data base using *S. pombe* Cdm1. The open reading frame of human EST AA402118 encoded a polypeptide of 107 amino acids with a predicted molecular mass of 12.4 kDa, consistent with the experimental findings. p12 is 25% identical to *S pombe* Cdm1. Both of the 25-kDa polypeptide sequences matched the hypothetical KIAA0039 protein sequence, recently identified as the third subunit of pol δ. Western blotting of immunoaffinity purified calf thymus pol δ revealed the presence of p125, p50, p68 (the KIAA0039 product), and p12. With the identification of p12 mammalian pol δ can now be shown to consist of four subunits. These studies pave the way for more detailed analysis of the possible functions of the mammalian subunits of pol δ.

DNA polymerase δ (pol δ) is the key polymerase that is involved in the replication of chromosomal DNA in eukaryotic cells. Studies of the *in vitro* replication of SV40 DNA have established that pol δ plays a central role in mammalian DNA replication (1). Proliferating cell nuclear antigen (PCNA),[1] the molecular sliding clamp of pol δ, is a processivity factor for pol δ and ε (2). PCNA was first identified as an activating factor for pol δ (3, 4) and is essential for replicative DNA synthesis. Several other factors have been identified as being required for SV40 DNA replication. Replication factor C (also known as activator-1) binds to the primer-template terminus, following which it recruits PCNA and then pol δ (5, 6) onto the DNA template. Replication Protein A, the single stranded DNA-binding protein, is involved in both initiation and elongation and also stimulates pol δ activity when replication factor C and PCNA are present (7, 8). The current view of DNA replication at the replication fork is that the pol δ complex is responsible for synthesis of the leading strand and that pol δ also participates in synthesis of the lagging strand (1). DNA polymerase α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, and the actual elongation of the primers is performed by DNA polymerase δ in a process that requires "polymerase switching" (9). Additional proteins, including topoisomerase and helicase activities, are also involved in the movement of the replication fork (1).

Mammalian pol δ has been rigorously isolated by conventional methods as a heterodimer consisting of two subunits, p125 and p50 (3). The subunit structure of pol δ has been the focus of recent investigations in yeast, and these have led to the identification of additional subunits. In *Schizosaccharomyces pombe*, pol δ is believed to consist of at least four subunits: a large catalytic subunit (Pol3) and three smaller subunits (Cdc1, Cdc27, and Cdm1) (10, 11). Pol δ purified from *Saccharomyces cerevisiae* is composed of three subunits: Pol3p, Pol31p/Hys2, and Pol32p (12–14). The pol δ core purified from calf thymus consists of two subunits: p125 and p50 (3). However, we have found that recombinant p125 catalytic subunit alone can only be stimulated by PCNA by 2-fold at most, while the overexpressed p125/p50 heterodimer is stimulated much less than pol δ purified by immunoaffinity chromatography (15, 16). These findings suggest that additional factor(s) which may be removed during protein purification are required for a full PCNA response in our assay. This is consistent with the hypothesis that mammalian pol δ may also contain additional subunits.

Using the proteomics approach, by peptide sequencing of polypeptides associated with the core pol δ in highly purified preparations isolated by p125 immunoaffinity chromatography, we have previously identified a 68-kDa polypeptide that is encoded by KIAA0039 and which is associated with the pol δ core. The p68 polypeptide is the third subunit of mammalian pol δ (17). Using a combination of proteomic approaches and GenBank searches, we have identified a novel subunit of pol δ that is the mammalian homologue of Cdm1, which in *S. pombe* is the fourth subunit of pol δ. Mammalian pol δ may thus consist of at least four subunits.

EXPERIMENTAL PROCEDURES

Materials—cDNA AA402118 was obtained from ATCC (Rockville, MD). Calf thymus tissue was obtained from Animal Technologies (Tyler, TX). Q-Sepharose, SP-Sepharose, heparin-Sepharose, Mono Q columns, and Mono S columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Purification of Calf Thymus Pol δ—The immunoaffinity purification was performed as described previously by Jiang *et al.* (18).

Conventional Purification of Calf Thymus Pol δ—The following buffers were used: lysis buffer consisted of 50 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, 10 mM benzamidine. TGEED buffer consisted of 50 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; pol, polymerase; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

TABLE I
Purification of calf thymus DNA polymerase δ

Fraction	Total volume	Protein	Total units	Specific activity
	ml	*mg*		*units/mg protein*
DE 52	3,615	18,075	397,000	22
Q-Sepharose	470	2,585	295,000	113
SP-Sepharose	176	722	468,000	648
Mono Q	34	76.5	56,000	736
Heparin-Sepharose	17	12.2	39,000	3,211
Mono S	3.3	2.1	9,400	4,500
Source Q15	3.0	0.15	900	6,000
Superdex 200	0.8	0.022	200	8,900

5% glycerol. KGEED buffer consisted of 20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.

All steps were carried out at 0–4 °C. Pol δ activity was assayed using poly(dA)/oligo(dT) as a template (19). Eight hundred grams of frozen calf thymus tissue in 4 liters of lysis buffer were homogenized in a Waring blender. The suspension was centrifuged at 5,000 rpm at 4 °C for 1 h and filtered through glass wool. The supernatant was mixed with 1.5 liters of DE52-cellulose equilibrated with TGEED buffer and stirred for 30 min. The mixture then was filtered through a Buchner funnel. The DE52-cellulose was washed with TGEED and the pol δ activity was stripped off with 20% ammonium sulfate in TGEED buffer.

The 3.5 liters of DE52-cellulose fraction were precipitated by the addition of 320 g/liter of ammonium sulfate. The suspension was stirred for 30 min, kept on ice for an additional 30 min, and then centrifuged at 10,000 × *g* for 45 min. The precipitate was resuspended in TGEED and dialyzed against TGEED buffer containing 50 mM NaCl with two changes and applied on to a 70-ml Q-Sepharose column. The bound proteins were eluted with a linear gradient of 50–750 mM NaCl in TGEED. The peak fractions containing pol δ activity were pooled and dialyzed against KGEED buffer containing 25 mM KCl. The Q-Sepharose fraction was loaded on to a 50-ml SP-Sepharose column. Pol δ was eluted with a linear gradient of 25–650 mM KCl in KGEED. The fractions containing enzyme activity were pooled and applied to a 10-ml Mono Q column, which was equilibrated with TGEED buffer containing 25 mM NaCl. The column was washed with 40 ml of TGEED buffer containing 25 mM NaCl. The activity was eluted with a gradient of 25–650 mM NaCl in 100 ml of TGEED at a flow rate 0.4 ml/min. The Mono Q fractions were pooled and dialyzed against TGEED buffer containing 25 mM NaCl and applied to a 5-ml heparin-Sepharose column. The column was washed with 2 column volume of TGEED containing 25 mM NaCl and eluted with a 50-ml gradient of 25–750 mM NaCl in TGEED at a flow rate 0.5 ml/min.

The heparin-Sepharose fraction (17 ml) was dialyzed against two changes of KGEED buffer containing 50 mM KCl and loaded onto a 1-ml Mono S column equilibrated with KGEED buffer. The column was washed with 5 ml of KGEED buffer and then eluted with a 20-ml linear gradient of KGEED buffer from 50 to 700 mM KCl. The active fractions were combined and dialyzed against TGEED buffer until the conductivity reached that of TGEED containing 50 mM NaCl. The fraction was applied to a Source Q15 column. The enzyme was eluted with a linear gradient of 50–650 mM NaCl in TGEED. The fractions with enzyme activity were pooled (3.0 ml) and concentrated to 270 μl using Centricon 30 (30,000 MW cutoff, Amicon). The concentrated enzyme (270 μl) was chromatographed on a FPLC Superdex 200 column equilibrated with TGEED buffer containing 150 mM NaCl. Fractions above 50% of the maximum peak of activity were pooled.

Protein Sequence Analysis—Polypeptide bands excised from a Coomassie Blue-stained gel were used for protein sequence analysis by the Harvard Microchemistry Facility using a microcapillary reverse-phase high performance liquid chromatography nano-electrospray tandem mass spectrometry (μLC-MS-MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

Antibodies—Peptide rabbit polyclonal antibodies against p12/hCdm1 and p68 were generated from a commercial source (SynPep, Dublin, CA) and purified by a peptide affinity column made from the same peptide antigen. For p12, the peptide contains amino acid residues 77 to 94 of p12 (H_2N-GLEPPPEVWQVLKYHPGD-COOH). For p68 (encoded by KIAA0039) the 19-amino acid peptide from near the extreme N terminus of p68 was used (H_2N-TDQNKIVTYKW-LSYTLGVH-COOH).

Western Blot Analysis—Proteins were transferred to 0.45 μM nitrocellulose membranes (Bio-Rad) after SDS-PAGE in transfer buffer (25 mM Tris-HCl, 192 mM glycine containing 10% v/v methanol) in a Genie blotter (Idea Scientific, Minneapolis, MN) for 75 min for 0.8-mm thick gels using a constant voltage of 12 volts. The membrane was incubated in TBST buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% Tween 20) containing 5% fat-free dry milk for 1 h at room temperature and washed briefly with TBST. The membrane was incubated with primary antibody for 1 h at room temperature or overnight at 4 °C. The membrane was washed 3× with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Pierce, Rockford, IL) for 1 h. The membrane was washed 3× with TBST. SuperSignal West Pico Chemiluminescent Substrate was used for signal production (Pierce) and the signal was captured on a Blue Bio film (Denville Scientific, Metuchen, NJ) after exposure for 15 s to 30 min and developed.

RESULTS

Demonstration That p68 and p12 Are Subunits of Mammalian Pol δ—We have previously devised a conventional procedure for the rigorous isolation of the pol δ core enzyme containing p125 and p50 (3). In order to isolate a multisubunit form of mammalian pol δ, a new purification scheme was devised, which allowed the isolation of pol δ core that retained associated polypeptides. This involved successive chromatographies on DE52, Q-Sepharose, SP-Sepharose, Mono Q, heparin-Sepharose, Mono S, Source Q15, Superdex 200 supports, including four FPLC chromatography steps (Mono Q, Mono S, Source Q15, and Superdex 200). Table I shows the purification of pol δ by this means. The specific activity of the preparation (about 9,000 units/mg) was comparable with that of pol δ purified by immunoaffinity chromatography (18). Review of a number of preparations isolated by the latter method gave an average specific activity about 10,000 units/mg, with a PCNA stimulation of 20–40-fold. The PCNA stimulation of 30-fold was found for the preparation obtained by the new procedure. This is similar to that of the immunoaffinity purified enzyme. The average specific activity of the purified recombinant pol δ heterodimer in our hands is about 2000 units/mg, with maximum PCNA stimulations of 6–10-fold. These results indicate that rigorously purified pol δ p125/p50 heterodimer has lost a significant fraction of its ability to respond to PCNA.

The final purification step used was FPLC gel filtration on Superdex 200. Calibration of the column showed that the peak of pol δ activity was eluted at a position indicating a much higher molecular weight (280,000) than can be accounted for by the two-subunit core. The Coomassie Blue staining of this pol δ complex is shown in Fig. 1. There were six major bands in the peak fractions of pol δ from fractions 48 to 50; these were of 125 kDa, 50 kDa, a doublet at about 25 kDa and a doublet at about 12 kDa. These polypeptide bands were excised from the Coomassie Blue-stained gel and sequenced at the Harvard Microchemistry Facility using LC/MS/MS methods. The sequencing results are displayed in Table II. Both the 25-kDa polypeptides were identified as the proteolytic products of KIAA0039, which was recently found to be associated with pol δ by a PCNA overlay assay (17). The KIAA0039 product was also eluted with pol δ from a PCNA affinity column (20). Our data support the view that p68 is the mammalian third subunit of pol δ but

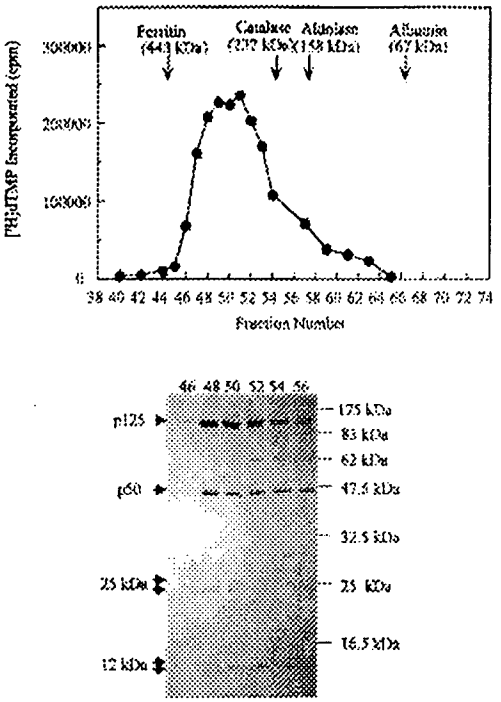

FIG. 1. **Chromatography of the purified calf thymus pol δ complex on Superdex 200.** The figure shows the elution profile of the calf thymus pol δ at the final purification step ("Experimental Procedures"). *Upper panel*, the activity of pol δ in the fractions of Superdex 200 gel filtration chromatography was assayed using poly(dA)/oligo(dT) as the template in the presence of PCNA. *Lower panel*, the peak fractions from the Superdex 200 column were separated on a 10% SDS-polyacrylamide gel and stained for protein with Coomassie Blue. 25-kDa upper and lower and 12-kDa upper and lower, as well as the p125 and p50 pol δ core subunits are marked by *arrows*.

TABLE II
Peptide sequences data from calf thymus pol δ complex

Identity	Peptide mass on SDS-PAGE	Peptide sequences obtained
Pol δ p125	125 kDa	IFEPILGEGR
		DPGSAPSLGDRVPYVIISAAK
Pol δ p50	50 kDa	FLGTSGQNVSDIFR
		AMPLQPSILR
		TQAASVEAVK
Pol δ p68[a] (KIAA0039)	25-kDa upper	VAVVREDKLEAVK
		QMLYDYVER
Pol δ p68[a] (KIAA0039)	25-kDa lower	WLSYTLGVHVNQAK
		FSAIQCAAAVPR
		DSGPLFNTDYDILK
		GIMGMFASK
		QMLYDYVER
Keratin 16	12-kDa upper	ADLEMQIENLR
Pol δ p12	12-kDa lower	QFDLAWQYGPCTGITR

[a] The protein has a size of 68 kDa present in p125-immunoaffinity purified calf thymus pol δ. In this purification scheme, it was proteolysed into two 25-kDa species.

indicate that it is highly susceptible to proteolysis.

The sequence of the upper 12-kDa band showed that this was derived from keratin. The second 12-kDa polypeptide was found to be a novel protein. The partial sequence obtained from this protein was QFDLAWQYGPCTGITR (Table II). This sequence was searched against the known protein data bases, which did not provide a match. A tBlastn search of the EST data base showed a match with one human EST sequence, AA402118, which, however, did not have a well defined open reading frame. Concurrently, the *S. pombe* Cdm1 protein sequence, which represents the fourth subunit of pol δ, was used for a tBlastn search at NCBI. This also retrieved the human EST sequence, AA402118. The EST cDNA clone was obtained from ATCC and was resequenced and corrected. The corrected sequence contained an open reading frame that encoded a protein of 107 amino acid residues, with a predicted molecular mass of 12.4 kDa. This protein was designated as p12. The corrected DNA sequence has been deposited in GenBank with the accession number AJ179890 (Fig. 2). The peptide sequence obtained from p12 shows a perfect match with residues 51–65 of the open reading frame of AJ179890.

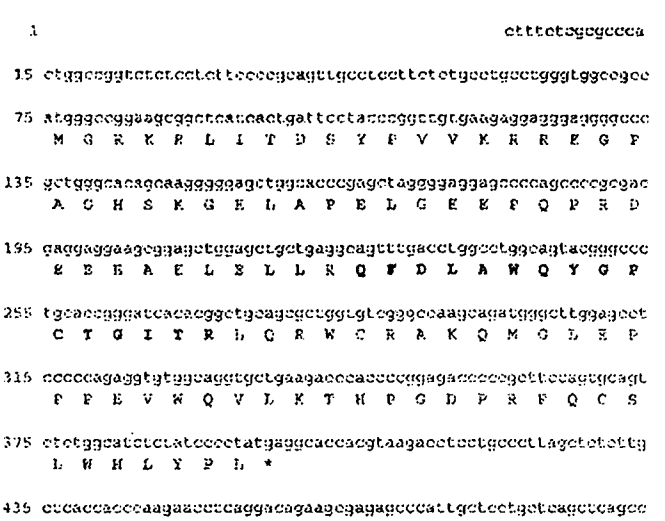

FIG. 2. **Nucleotide sequence and predicted amino acid sequence of the smallest subunit of mammalian DNA polymerase δ.** This figure shows the nucleotide sequence of the human EST AA402118 which was corrected by resequencing (AJ179890). The insert of the human EST A402118 in the cloning vector pT7T3D is 512 base pairs, and encodes a protein of 107 amino acids. The amino acid sequence of the peptide derived from the 12-kDa polypeptide in the calf thymus pol δ preparation (Fig. 1) is shown in ***bold***.

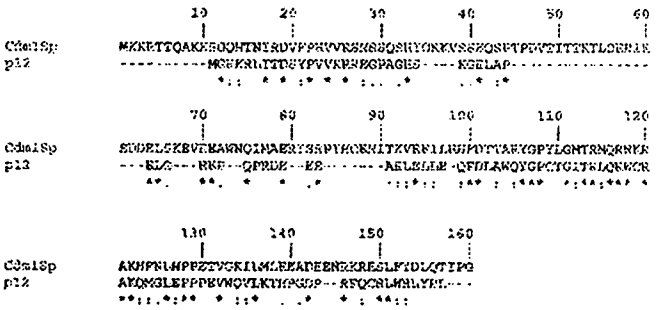

FIG. 3. **Alignments of human p12 with *S. pombe* Cdm1.** Human p12 (AJ179890) and *S. pombe* Cdm1 (emb AJ006032) were aligned with the Clustal 1.74 program. The identity between these two proteins is 25% and the similarity is 39%.

These results indicate that p12 is a likely human homologue of the *S. pombe* Cdm1 protein, which has been reported to be the fourth subunit of *S. pombe* pol δ (10, 21). The *S. pombe* Cdm1 protein has a calculated molecular mass of 18.5 kDa and an apparent size of 22 kDa on SDS-PAGE, and is significantly larger than human p12. Sequence alignments were performed to assess the possible relationships between these two proteins. Protein sequence alignment indicates that the identity between p12 (107 residues) and *S. pombe* Cdm1 (160 residues) is 25% and the similarity is 39% (Fig. 3). It can be seen that the main region of identity of p12 is with the C-terminal half of *S. pombe* Cdm1. Alignment of amino acid residues 96- 142 of Cdm1 with

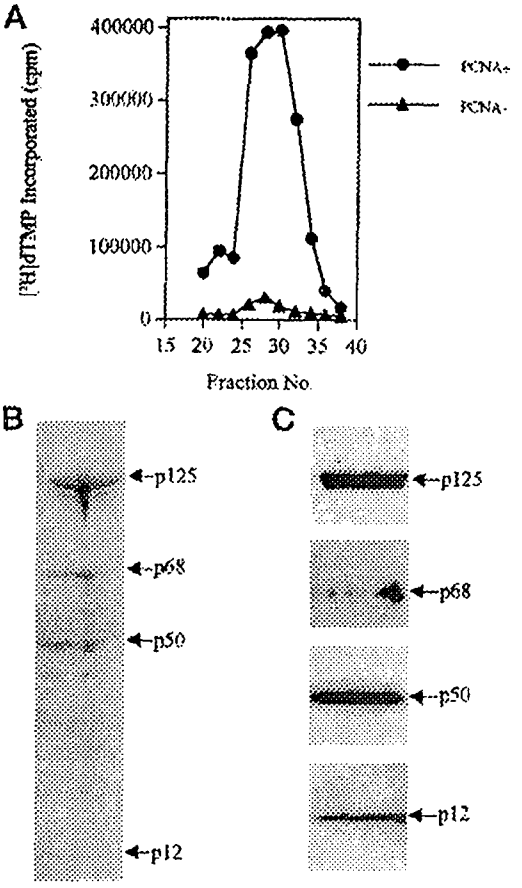

FIG. 4. **Western blot analysis of pol δ subunits purified by p125-immunoaffinity chromotography from calf thymus.** Calf thymus pol δ was purified through DE52, phenyl-agarose, and p125 immunoaffinity column chromatographies in the presence of protease inhibitors (18). *Panel A,* activity assay of the fractions eluted from the immunoaffinity column using poly(dA)/oligo(dT) as the template in the absence (*closed triangles*) and presence (*closed circles*) of PCNA. *Panel B,* Coomassie Blue staining of peak fraction number 28. *Panel C,* Western blotting of peak fraction number 28 using monoclonal antibody 78F5 against p125, 13D5 against p50, and rabbit peptide polyclonal antibodies against p68 and p12.

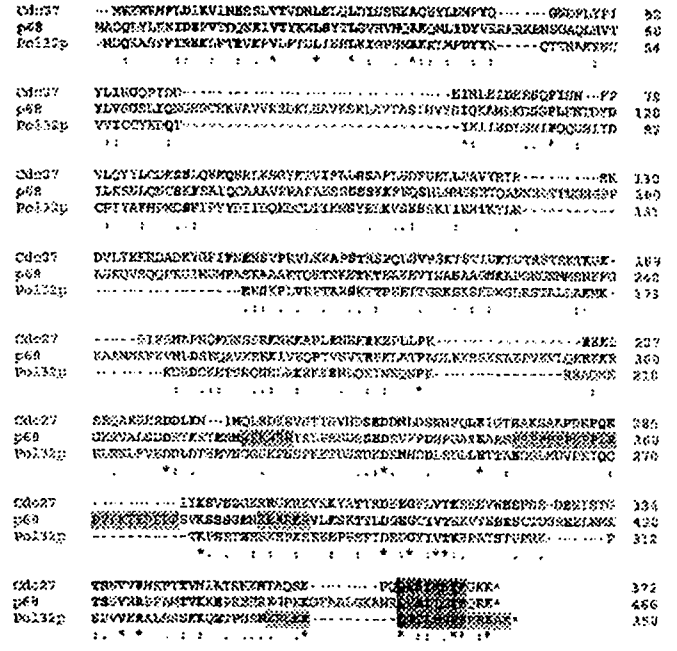

FIG. 5. **Multiple sequence alignment of p68, Pol32p, and Cdc27.** p68 (the protein product of KIAA0039, BAA05039) with *S. pombe* Cdc27 (P30261) and *S. cerevisiae* Pol32p (CAA89571) were analyzed using the Clustal 1.8 program. The PCNA binding motif is highlighted in *dark gray*. The putative nuclear localization signals of p68 and Pol32p are highlighted in *light gray*. The unique proline-rich motif in p68 is also highlighted.

TABLE III
Summary of pol δ subunits

DNA polymerase δ	Mammalian	*S. pombe*[a]	*S. cerevisiae*[b]
The catalytic subunit	p125	125 kDa, Pol3	125 kDa, Pol3p
The second subunit	p50	55 kDa, Cdc1	58 kDa Pol31p
The third subunit	p68[c]	54 kDa, Cdc27	55 kDa Pol32p
The fourth subunit	p12[d]	22 kDa, Cdm1	Not found

[a] Isolated from *S. pombe* (10).
[b] Reconstituted as a three-subunit enzyme (13).
[c] Identified as bovine p68 (encoded by KIAA0039) in p125 immunoaffinity purified pol δ (17). Isolated by PCNA affinity chromatography of mouse FM3A cell extracts (20).
[d] Identified in this study.

residues 48 to 94 of p12 shows that there is a 44% identity. This degree of similarity is sufficient for p12 to be regarded as the mammalian homologue of *S. pombe* Cdm1. Taken together with the sequence identification of the p12 and its co-purification with the calf thymus pol δ core through eight chromatography procedures, these findings provide strong evidence for the identification of p12 as a novel subunit of mammalian pol δ.

Western Blot Analysis of Immunoaffinity Purified Pol δ—We had previously shown that pol δ isolated by immunoaffinity chromatography contains the pol δ core in association with a number of other polypeptides (18), and also displayed a much higher molecular weight than could be accounted for by the core on gel filtration analysis (22). The failure to observe p12 in these studies could be due its small size and the fact that it migrated close to the dye front under the conditions used. A preparation of pol δ was purified from calf thymus using immunoaffinity chromatography (18) and the preparation was assessed for the presence both of the p68 and p12 subunits. The presence of these two polypeptides on SDS-PAGE gels of the preparation are shown in Fig. 4. Polypeptides corresponding to 68 and 12 kDa were prominent components of the preparation, and their identity as the p68 and p12 polypeptides was confirmed by Western blotting (Fig. 4). Thus, the presence of all four subunits of pol δ (p125, p50, p68, and p12) were demonstrated in this preparation (Fig. 4). The KIAA0039 product in the Western blot was 68 kDa.

p68 Is the Third Subunit of Mammalian Pol δ, the Homologue of S. pombe Cdc27 and S. cerevisiae Pol32p—The p68 sequence has a conserved $p21^{Waf1}$-like PCNA binding motif at the extreme C terminus, as does *S. pombe* Cdc27 and *S. cerevisiae* Pol32p, the yeast third subunits of pol δ (13). The p68 sequence encoded by KIAA0039 was aligned with the sequences of Cdc27 and Pol32p (Fig. 5). Analysis of the alignments showed that p68 shares little sequence identity with Cdc27 and Pol32p. The only sequence conservation was the C-terminal PCNA binding motif in these three sequences. p68 and Pol32p both have nuclear localization motifs. p68 also has an unique proline-rich motif. Pairwise alignments using the Clustal W 1.8 program show that between Pol32p and Cdc27, Pol32p and p68, or Cdc27 and p68 there is only 15 to 16% sequence identity (not shown). However, evaluation of the significance of the alignment score for p68 with Cdc27 using the PRSS program provided a score of 0.4, *i.e.* the alignment score (% identity) would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 times in 100 attempts. This indicates that the similarity between these two proteins is significant.

TABLE IV
The third subunits and putative third subunits of pol δ

Organism[a]	Accession No.	Protein	Length	PCNA binding motif
1	BAA05039	p68	466 amino acids	^{396}QVSITGFFQRK^{466}
2	CAA98958.1		445 amino acids	^{435}NSMITSFFKT^{445}
3	AAD30571.1		509 amino acids	^{499}QGNIMSFFKKV^{509}
4	AAD38629		431 amino acids	^{420}QAGIMNFFSKK^{431}
5	P30261	Cdc27	372 amino acids	^{362}QKSIMSFFGKK^{372}
6	CAA89571	Pol32p	350 amino acids	^{338}QGTLESFFKRKA^{350}

[a] 1. *Homo sapiens*; 2. *C. elegans*; 3. *A. thaliana*; 4. *D. melanogaster*; 5. *S. pombe*; 6. *S. cerevisiae*.

DISCUSSION

The thrust of the earlier studies of pol δ was the rigorous isolation of the enzyme which culminated in the isolation of a two-subunit enzyme, containing a catalytic subunit of 125 kDa and a second subunit of 50 kDa (3, 23). The major difficulties in this process were the small amounts of protein available and the likelihood of proteolysis using extensive purification schemes due to the fragile nature of the mammalian system compared with other systems, *i.e.* prokaryotic or lower eukaryotes such as yeast. Recently, expression systems for the p125 (15, 24, 25), the p50 subunit (16), and the recombinant heterodimer have been developed (26). The p50 subunit has no known enzymatic functions, but has been shown to be required for the response of the p125 subunit to PCNA (16, 26). In this study we have shown for the first time the isolation of a four-subunit mammalian pol δ enzyme. This newly isolated pol δ contains the third subunit p68 and a previously unknown subunit, p12. The latter two are the mammalian homologues of *S. pombe* Cdc27 and Cdm1, respectively.

The association of p68 and p12 with pol δ was demonstrated by their isolation with p125 and p50 from calf thymus through extensive purification involving multiple conventional column chromatographies as well as by immunoaffinity chromatography. The conventional procedure included several FPLC steps including gel permeation chromatography. The strong association of the p68 and p12 polypeptides with the pol δ core provide very strong evidence for the proposal that these represent subunits of pol δ. p68 has also been isolated from mouse cell extracts using a PCNA affinity column in association with the pol δ core consisting of the p125 and p50 subunits (20). There are extensive technical problems associated with the identification of subunits of mammalian pol δ. As encountered in our studies, these include the susceptibility of the p68 polypeptide to proteolysis and the difficulties of isolation of pol δ from animal tissues to study stoichiometries of pol δ subunits in native enzyme preparations. Nevertheless, in these studies it is demonstrated that it is possible to rigorously isolate pol δ from calf thymus in a form which retains the p68 and p12 polypeptides. A key difference in the new method from the older procedure (18) was the avoidance of single-stranded DNA cellulose chromatography.

The identification of the fourth subunit of pol δ in mammalian systems now provides a parallel for the situation found in yeast. A comparison of the subunit structures of pol δ from the mammalian and the two yeast models is shown in Table III. The catalytic subunit of mammalian pol δ is strongly conserved in evolution, and shares a high degree of homology with the corresponding catalytic subunits in *S. pombe* and *S. cerevisiae*, the identity being greater than 48% (27). The p50 subunit is less conserved than the catalytic subunit, the identity between p50 and *S. pombe* being 33% (11). Furthermore, the finding that PCNA from human or yeast origin can activate the heterologous pol δ preparations strongly suggests that the pol δ complex is functionally conserved to a high degree (28). The functions of these subunits are still incompletely understood. The third subunit of *S. pombe* pol δ was only recently identified (10) and is encoded by the *cdc27*$^+$ gene, which is needed for the transition of G_2/M in the cell cycle (11). The third subunit of *S. cerevisiae* pol δ is Pol32p, was isolated and identified in 1998. It was proposed as a candidate for dimerization factor of pol δ (13) based on the finding that the recombinant three-subunit enzyme could be shown to behave as a dimer on gel filtration (13). In addition, Pol32p was found to interact with the pol α catalytic subunit by the yeast two-hybrid method (29). These results suggest that Pol32p can (*a*) dimerize pol δ at the replication fork, and (*b*) provide a means for the proposed "polymerase" switch at the lagging strand through the interaction with pol α as suggested by Waga *et al.* (1).

p68, the mammalian homologue of *S. pombe* Cdc27, KIAA0039 was isolated from a PCNA affinity column (20) and from an immunoaffinity column of pol δ p125 (17). The third subunits of pol δ share a very low degree of similarity. In fact, Blast searches with Cdc27 failed to identify either p68 or Pol32p. tBlastn searches using Pol32p only identified a *Drosophila melanogaster* third subunit of pol δ. Similarly, using p68 the putative *Caenorhabditis elegans* and *Arabidopsis thaliana* third subunit of pol δ were identified (Table IV). As already noted ("Results"), the third subunits of human, *S. pombe*, and *S. cerevisiae* are poorly conserved, although the relationships based on the alignments can be shown to be significant. The third subunits of pol δ from different species all contain a putative p21^{waf1}-like PCNA binding motif (30, 31) at the extreme C terminus. An important aspect of the third subunit is that it interacts with PCNA, and also with the yeast p50 homologues (11, 13, 32). The ability of p68 to bind to PCNA (17, 20) may account for the loss of sensitivity to PCNA shown by pol δ p125/p50 heterodimer. In addition, all share in common a high content of charged amino acids which ranges from 29 to 35%. The calculated isoelectric points for these proteins are all basic, with the exception of the *S. pombe* Cdc27, which has an acidic isoelectric point. This common property suggests that p68 is likely to have an extended structure in solution, which is also consistent with its apparent liability to proteolysis. A third property of the third subunit may be an ability to interact with the p50 second subunit, which has been demonstrated in *S. pombe* and *S. cerevisiae* (11, 13) and also in mammalian pol δ.[2] One speculative function of p68 may be to act as a linker protein between p50 and PCNA, which would provide additional stabilization of the pol δ-PCNA interaction. This possibility is consistent with the higher sensitivity to PCNA of the pol δ preparations which contain p68 compared with that of the heterodimer.

Thus far, the fourth subunit has only been identified in mammalian sources in this present work, and previously as Cdm1 in *S. pombe*. Interestingly, data base searches have failed to identify a homologue in *S. cerevisiae*, despite the fact that its entire genome has been cloned. This may be due to a lack of evolutionary conservation. The functions of this newly

[2] L. Liu and M. Y. W. T. Lee, unpublished observations.

described subunit also remain to be determined.

In summary, this work provides evidence for the identification of a novel subunit, p12, as a component of mammalian pol δ, as well as evidence for the isolation of pol δ in a form that contains the core heterodimer in association with both p12 and the third subunit, p68.

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A Novel PCNA-Binding Motif Identified by the Panning of a Random Peptide Display Library†

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ABSTRACT: Proliferating cell nuclear antigen (PCNA) has recently been identified as a target for the binding of proteins involved in DNA replication, DNA repair, and cell cycle control. The interactions between PCNA and a number of these proteins are known to be mediated by a conserved peptide motif. In this study, a random peptide library in which peptide sequences are displayed on the *E. coli* bacterial flagellin protein was screened for PCNA-binding sequences. Analysis of the retrieved peptide sequences verified the presence of the known PCNA-binding motif. In addition, a second, larger group of peptides containing a different consensus sequence for PCNA binding was discovered. This sequence was found to be present on DNA polymerase δ, and a peptide conforming to this sequence was demonstrated to bind to PCNA. Database search and analysis show that many proteins contain the second consensus sequence. These include proteins that are involved in DNA replication, repair, and cell cycle control. The demonstration of this second PCNA-binding motif may provide a basis for identifying and experimentally testing specific proteins for the structural basis for PCNA binding.

Proliferating cell nuclear antigen (PCNA) is a highly conserved eukaryotic protein that functions in DNA replication as a molecular sliding clamp that permits highly processive synthesis by DNA polymerase δ (pol δ) (*1*). PCNA, originally identified as a processivity factor for pol δ, has been intensively investigated both in terms of its structure and in terms of its role in cellular processes. Expression of recombinant human PCNA and its physicochemical characterization established that it is a trimeric protein (*2*), and the crystal structures of both yeast PCNA (*3*) and human PCNA (*4*) have shown that they are structurally and functionally homologous to the T4 gene 45 protein and the β subunit of *E. coli* DNA polymerase III holoenzyme DNA sliding clamps (*5, 6*). PCNA interacts with the clamp loader replication factor C (RFC) (*7*−*9*), DNA polymerase δ (*10, 11*), replication endonuclease FEN-1 (*12, 13*), and DNA ligase I (*14*) and plays a role in both leading- and lagging-strand DNA synthesis at the replication fork. During the past several years, it has been shown that PCNA also interacts with proteins involved in cell cycle progression and DNA repair. The DNA repair endonuclease XPG (*15*), the major nuclear uracil DNA−glycosylase (UNG2) (*16*), and the mismatch repair protein MSH2−MSH3 heterodimer (*17*) interact with PCNA. PCNA also binds to DNA (cytosine-5) methyltransferase (MCMT) (*18*), cyclin D (*19*), the cell cycle regulated nuclear protein Gadd45 (*20, 21*), and the cell cycle regulatory protein p21 (*22, 23*). Thus, PCNA, by virtue of its functions as a sliding clamp, may have multiple cellular functions associated with processes involving DNA modification or synthesis. Moreover, it may be a target for cell cycle regulation, as evidenced by its interactions with cyclin D, p21, p57 (*24*), and GADD45 (*20, 21*).

The ability of PCNA to interact with multiple protein partners having disparate structures is explicable at least in part through the existence of a PCNA-binding motif that is present on a number of its binding proteins (*25*). This conserved PCNA-binding motif was termed the PCNA interaction protein box (PIP-box) (*26*). An alignment of these binding motifs shows that it consists of the sequence Q-x-x-(h)-x-x-(a)-(a) (where "h" represents residues with moderately hydrophobic side chains, e.g., L, I, M; "a" represents residues with highly hydrophobic, aromatic side chains, e.g., F, Y; and "x" is any residue) (*25, 26*). Proteins which exhibit such a PCNA-binding PIP-box include p21, Fen1, XPG, and Dacapo protein, which is a cyclin-dependent kinase inhibitor (*27, 28*), and the *Pogo* transposon (*29*). In the case of p21, the structural basis for the interaction of this motif with PCNA has been determined at the atomic level by crystallographic analysis of a p21 peptide−PCNA complex (*4*). The peptide consisted of residues 139-GRKRRQTSMTDFYHS-KRRLIFS-160, and harbors the sequence QTSMTDFY which conforms to the PIP-box. The interaction of the peptide with PCNA involves three general features: interaction with a large hydrophobic pocket, a small hydrophobic pocket, and an extended interaction with the interdomain connector loop of PCNA (*4*). The residues in the PIP-box interact with the large hydrophobic pocket, and involve the two aromatic residues of the PIP-box. Thus, in the case of p21, the PIP-box represents only part of the protein−protein interface with PCNA. A 20 amino acid sequence that exists in both the N-terminal region of DNA ligase I and the large subunit of

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RFC has been proposed to function as a replication factory targeting sequence (RFTS). This sequence also binds to PCNA (*30*).

Because PCNA appears to be involved in the binding of a number of protein partners, the question of whether it contains sites for recognizing other peptide motifs can be raised. In addition, the short sequence of the PIP-box, and the fact that only three of eight residues in this sequence are conserved, suggests that its presence in a given protein does not necessarily provide strong evidence that the protein binds PCNA. Conversely, not all the known PCNA-binding proteins contain sequences which might conform to the PIP-box motif, e.g., cyclin D and pol ϵ. Mutational analyses of PCNA have shown that mutations in different regions of PCNA can result in differential effects with a given partner [reviewed by Tsurimoto (*31*)]. To obtain more information on the peptide motifs that are recognized by PCNA, a random peptide library was screened against PCNA. The results provide evidence that PCNA may bind to a second class of peptides in addition to those conforming to the PIP-box, and further verify the existence of the conserved motif (PIP-box).

EXPERIMENTAL PROCEDURES

Materials. The FliTrx random peptide library was obtained from Invitrogen (San Diego, CA). The Sephacryl S-200 column and Mono P column for PCNA purification, the activated CH-Sepharose, the T7 Sequenase version 2.0 DNA sequencing kit, the protein biotinylation system, and the ECL chemiluminescence detection reagents were purchased from Amersham−Pharmacia Biotech Inc. The QIA prep miniprep kit for plasmid isolation was obtained from QIAGEN. Pol δ synthetic peptides, C1 (1047-LEERFSRLWTQCQRCQGSL-HED-1068), C2 (1069-VICTSRDCPIFYMRKKVRKDLED-1090), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N2−1 (mutant of N2, 129-GVTDEGASVCCHIHGFAPYFY-149, F → A), N2−2 (mutant of N2, 129-GVTDEGFRVC-CHIHGFAPYFY-149, S → R), N2−3 (mutant of N2, 129-GVTDEGFSVACHIHGFAPYFY-149, C → A), N2−4 (mutant of N2, 129-GVTDEGFSVCCAIHGFAPYFY-149, H → A), N2−5 (mutant of N2, 129-GVTDEGFSVCCHI-AGFAPYFY-149, H → A), N2−6 (mutant of N2, 129-GVTDEGFSVAAHIHGFAPYFY-149, CC → AA), N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAPAAA-149, YFY → AAA), and N4 (276-RLKEKATQCQLEADVL-WSDV-295), were synthesized by the Protein Chemistry Core Laboratories (Miami, FL). The IMC medium used for growth of the *E. coli* peptide library was 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, pH 7.4, 0.2% casamino acids, 0.5% glucose, and 1 mM $MgCl_2$. TGD buffer consists of 50 mM Tris-HCl, 5% glycerol, 1 mM DTT, pH 7.8. The blocking solution consisted of 1% nonfat dry milk, 150 mM NaCl, 1% α-methyl mannoside, and 100 μg/mL ampicillin in IMC medium. The 1 L of RM medium contained 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, pH 7.4, 20 g of casamino acids, 10 mL of 50% glycerol, 100 μg/mL ampicillin, and 1 mM $MgCl_2$. The plating medium for RMG plates contained 15 g of agar in 1 L of RM medium containing 100 μg/mL ampicillin. TGEED buffer consists of 50 mM Tris-HCl (pH 7.8), 5% glycerol 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol.

Preparation of PCNA. Five milliliter overnight cell cultures of *E. coli* DHα containing the PCNA expression plasmid previously described (*2*) were used to inoculate 1 L cultures (Terrific media) and grown at 37 °C until the A_{600} reached 0.3. After addition of isopropyl-1-thio-β-D-galacto-pyranoside to a concentration of 0.3 mM, the cultures were grown for another 16 h at 28 °C. The cells were harvested, and the PCNA were purified as described by Zhang et al. with minor modifications (*2*). Before loading on a Sephacryl S-200 column for purification, the preparation was first purified by chromatography on a Mono P column.

Growth of the E. coli Peptide Library. The FliTrx random peptide library is based on the system described by Lu et al. (*32*). The *E. coli* strain GI826 containing pFliTrx with inserts was grown with shaking for 18 h at 25 °C in IMC medium containing 100 μg/mL ampicillin. The cultures (1×10^{10} cells) in 50 mL of IMC medium were induced to express the thioredoxin−flagellin fusion proteins containing the peptide inserts by adding ampicillin (100 μg/mL) and tryptophan (100 μg/mL) and grown for another 6−8 h at 25 °C.

Immobilization of PCNA on Culture Plates. The purified PCNA was dialyzed overnight with TGD buffer. PCNA was immobilized on 60 mm plastic Petri dishes by adsorption from 1 mL of PCNA solution (100−120 μg of PCNA/plate) with gentle agitation for 1 h. After washing the plate with 10 mL of sterile water, the immobilized plate was agitated for 1 h with 10 mL of blocking solution.

Panning of the Random Peptide Display Library. The panning technique was performed as described in the manufacturer's procedure with minor modifications. After the 6 h induction, the following were added to the 50 mL of induced cells: 0.5 g of nonfat dry milk, 1.5 mL of 5 M NaCl, and 2.5 mL of 20% α-methyl mannoside. The induced cells (10 mL) were added to the plate containing immobilized PCNA. The plate was rotated gently for 1 min at 50 rpm and allowed to incubate for 60 min at room temperature. The bacterial culture was decanted. The plate was washed by gentle agitation for 5 min with 10 mL of IMC medium containing 100 μg/mL ampicillin and 1% α-methyl manno-side, and the wash was repeated 4 times. The fifth wash was fully decanted, and the PCNA-bound bacteria were detached into a small volume of IMC medium by vortexing the plate for 30 s. The detached bacteria were grown as described above. The culture was then induced by growth with tryptophan-containing medium, and the cycle of panning was repeated. Nine rounds of panning were performed.

Biotin-Labeled PCNA. Purified recombinant PCNA (1 mg/mL) was dialyzed overnight in 40 mM sodium bicarbonate buffer, and concentrated on a Centricon-30 filter. PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester. One milliliter of PCNA solution (0.5 mg/mL) was incubated with 25 μL of biotinamido-caproate *N*-hydroxysuccinamide ester (5 mg/mL) at room temperature for 1 h with constant agitation. The reaction mixture was passed through a Sephadex G-25 column equilibrated with 5 mL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The conjugated PCNA was diluted 1000-fold in PBS containing 1% BSA and 0.2% sodium azide, and stored at 4 °C.

Preparation of Positive Clones for Analysis. After the ninth panning, the cultures were streaked onto RMG-Amp plates,

and single colonies were selected. Each colony was inoculated into 4 mL of RM medium, and grown at 30 °C to saturation with shaking (245 rpm) for 20 h until the A_{600} reached 2.0−3.0. The cultures (100 μL) were then inoculated with 3 mL of IMC medium containing 100 μg/mL ampicillin and 100 μg/mL tryptophan, and grown at 30 °C for 8 h until the cells reached mid-log phase (0.6 OD_{600}). The remainder of the noninduced cultures was saved at 4 °C for DNA isolations.

Overlay Blotting with Biotinylated PCNA. After induction, the cells were harvested and resuspended in 150 μL of SDS−PAGE sample buffer and heated at 100 °C for 5 min. Ten microliters of sample was loaded and subjected to electrophoresis on SDS−PAGE (10% acrylamide), and transferred to a nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk in PBST (1 × PBS, plus 0.05% Tween 20) for 2 h at room temperature with shaking followed by four washes of PBST for 10 min each. The blot was then incubated with biotinylated PCNA (0.5 μg/mL) at 4 °C overnight or for 2 h at room temperature with shaking. The blot was washed 5 times with PBST for 15 min each. It was subsequently incubated with streptavidin−horseradish peroxidase conjugate diluted in PBST (1:10 000) for 1 h at room temperature with shaking. After washing 5 times with PBST for 15 min each, the blot was developed using a chemiluminescence method (ECL detection system, Amersham).

DNA Sequencing. The remaining noninduced cultures from above were collected, and the pFliTrx plasmids were isolated. DNA sequencing was performed by the dideoxy chain termination method using the T7 Sequenase version 2.0 DNA sequencing system (Amersham). The primer used for DNA sequencing was the FliTrx forward sequencing primer, 5′-ATTCACCTGACTGACGAC-3′.

Sequence Analysis. The amino acid sequences of inserted peptides were analyzed by the Motif program at the GenomeNet Database (Kyoto University and the University of Tokyo, Japan). The protein sequence database used for search was SWISSPROT. The query pattern entered in the search was written in PROSITE format.

Dot Blot Analysis for the Interaction between Pol δ Synthetic Peptides and PCNA. About 5 μg of each peptide was dotted onto a nitrocellulose membrane and air-dried for 45 min at room temperature. After blocking the membrane with 5% nonfat dry milk in TBST (1 × TBS, plus 0.05% Tween 20) for 2 h at room temperature with shaking, the membrane was visualized by following the same procedures as for the PCNA overlay described above.

Preparation of Immobilized Peptides and Adsorption of PCNA on N4- or N2−6-Sepharose. Activated CH-Sepharose (0.25 g) was suspended in cold 2 mM HCl for 15 min and then washed 6 times with ice-cold 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (separated by centrifugation, 3000 rpm, 8 s). The gel was then mixed with the peptide solution (N4 and N2−6, 2.5 mg in 1 mL of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), and the suspension was rotated end-over-end overnight at 4 °C. The gel was blocked by incubation with 1 mL of 0.2 M glycine, pH 8, for 20 h at 4 °C and then washed 6 times with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (separated by centrifugation, 3000 rpm, 8 s). Purified recombinant human PCNA (40 μL, 0.1 μg/μL) was mixed with 40 μL of N4 or N2−6 coupled to CH-Sepharose. The suspension was mixed at 37 °C for 1 h. The beads were then centrifuged (3000 rpm, 8 s) and washed 7 times with 0.1 M $NaHCO_3$, 0.6 M NaCl, pH 8.3. After the final wash, the beads were resuspended in 40 μL of SDS−PAGE sample buffer and boiled for 5 min. The supernatant was then taken directly for SDS−PAGE and Western blot analysis using anti-PCNA antibody (*33*). The same procedure was used for coupling of bovine serum albumin, which was used as a control.

Enzyme Activity Assay. DNA polymerase δ activity was assayed with poly(dA)/oligo(dT) as a template-primer and [^{3}H]dTTP as the nucleotide donor. First, increasing amounts (1 mg/mL) of various synthetic peptides were added to 5 μL of PCNA (0.1 mg/mL) in each tube. The reactions consisted of either 0, 1, 2, 4, or 8 μL of the peptide(s). Each reaction was brought to the same volume by adding TGEED buffer. Each volume was mixed and allowed to react at room temperature for 1 h. Then, 5 μL of DNA polymerase and 45 μL of poly(dA)/oligo(dT) were added to each reaction. The reaction mixtures were incubated at 37 °C for 30 min. The samples were spotted onto DE81 filter paper circles and placed under a lamp for 10 min. Filters were washed 3 times in 0.3 M ammonium formate for 10 min each time and once with 95% ethanol for 5 min. Pol δ activity was determined by DNA binding to the DE81 filters as described by Lee et al. (*34*).

RESULTS

Panning of the FliTrx Random Peptide Display Library Using PCNA. The FliTrx random peptide display library used is based on the display of peptides on the flagella of *E. coli*. The FliTrx library has a diversity of 1.77×10^8 individual dodecapeptides; each is flanked by the sequences CPG and GPC at its N and C termini, respectively. These peptides are inserted into the active-site loop of thioredoxin, which is itself fused into the major flagellin protein of *E. coli*. After induction of the flagellin fusion protein in the cells, the peptides are thus displayed on the flagella. The screening consisted of consecutive rounds of panning on Petri dishes to which PCNA was immobilized. After nine rounds of panning (see Experimental Procedures), the plasmids from individual *E. coli* isolates were isolated and analyzed. To confirm that these isolates were indeed binding to PCNA, a PCNA overlay method was employed to detect thioredoxin−flagellin fusion proteins that bind to PCNA. This was done by SDS−PAGE of the *E. coli* proteins, followed by overlay blotting with biotin-conjugated PCNA. Figure 1 shows an example of this analysis in which eight isolates were tested. It can be seen that there are five isolates which show a positive overlay for PCNA binding to a protein of 68 kDa. The latter size is consistent with an expected size of the fusion protein since *E. coli* thioredoxin has a molecular mass of 12 kDa (*35*) and *E. coli* flagellin has a molecular mass of 60 kDa (*36*).

Analysis of the Peptide Sequences Isolated by Panning of the FliTrx Library. Eighty-five peptide inserts were obtained from the panning of the FliTrx library, and 49 of these were positive clones by the PCNA overlay method. Inspection of the insert peptide sequences of these positive clones shows that they did not fall into a single family. In Table 1, two groups of peptide sequences which clearly form two separate families are shown. The first of these (Group I, Table 1) contained those sequences that matched or were similar to

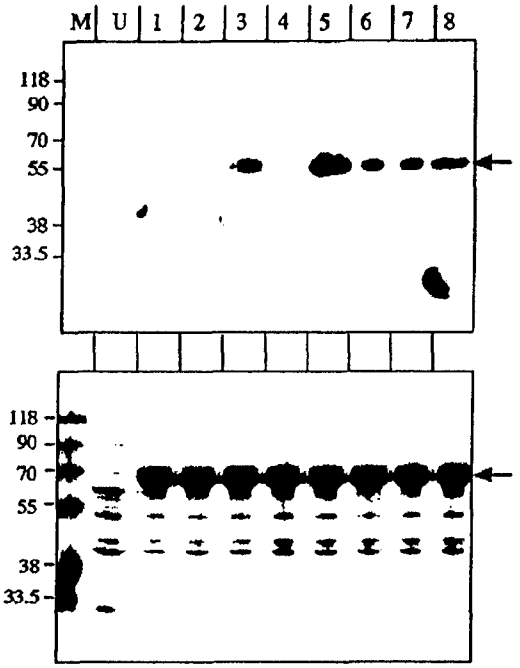


FIGURE 1: Binding of PCNA to thioredoxin−flagellin fusion proteins. The binding of PCNA to the thioredoxin−flagellin fusion proteins of the isolates obtained by panning of the random peptide library was tested by PCNA overlay of bacterial extracts (see Experimental Procedures). Induced cultures of isolated colonies were collected and resuspended in 150 μL of SDS−PAGE loading buffer. The samples were boiled for 5 min and then loaded onto SDS−PAGE (5 μL). After transfer to the nitrocellulose membrane, the blot was exposed to biotinylated PCNA and detected using streptavidin−horseradish peroxidase conjugate (see Experimental Procedures). The upper panel shows a representative overlay blot for eight individual isolates (lanes 1−8). The lane marked "M" contained the protein standards, and the lane marked "U" is that of a culture containing the FliTrx plasmid without induction. The lower panel shows the Coomassie blue stain for protein. The position of the 68 kDa thioredoxin−flagellin fusion protein is indicated by arrows.

the known PCNA PIP-box motif. Only the first five of the sequences in Group I can be obviously matched to the PIP-box. Sequences 8−6 and 9−15 conformed closely to the specifications of the PIP-box, Qxx(h)xx(a)(a) (h, residues with moderately hydrophobic side chains, e.g., L, I, M; a, residues with highly hydrophobic, aromatic side chains, e.g., F, Y; x, any residues). The next three possessed only a single aromatic residue, as has been found for the PIP-box for *Drosophila* p21 (*26*). The next two peptides (9−19 and 9−21) contained a pair of aromatic residues but without the conserved glutamine. PCNA overlay analysis confirmed that 9−19 reacts with PCNA. Members of the PIP-box motif which also do not contain the conserved glutamine are those present in p57 (*24*), and the PIP-box sequence that has been proposed to reside in the N2 region of the 125 kDa catalytic subunit of pol δ (*37*). Two additional sequences (7−3 and 7−2) are shown in Group I, but these can only be considered as marginal members of the PIP-box as they lack the aromatic residues.

The second group (Group II) only contained four members which are strongly related and are characterized by the presence of two or three aromatic residues. The search sequence Y-x(3)-[YT]-x(4)-W was used to search for related sequences in the SWISSPROT database using the

Table 1: Peptide Sequences from Screening of the FliTrx Random Peptide Library with PCNA as the Bait

Group I (PIP-Box motif)

8-6	K Q G R L A G F F K L G
9-15	F Q P A V G D F Y A S K
7-1a	A T Q H S K R A Y A V G
7-12	S Q R R S G P Y A A T L
9-47	A C V N R Q S R E D E E
9-19	T T G S G H G F Y K P G
9-21	M W R S E F Y T P P D E
7-3	E F Q C P R E A L K A H
7-2	D F Q C P R E A L K A H

Group II.

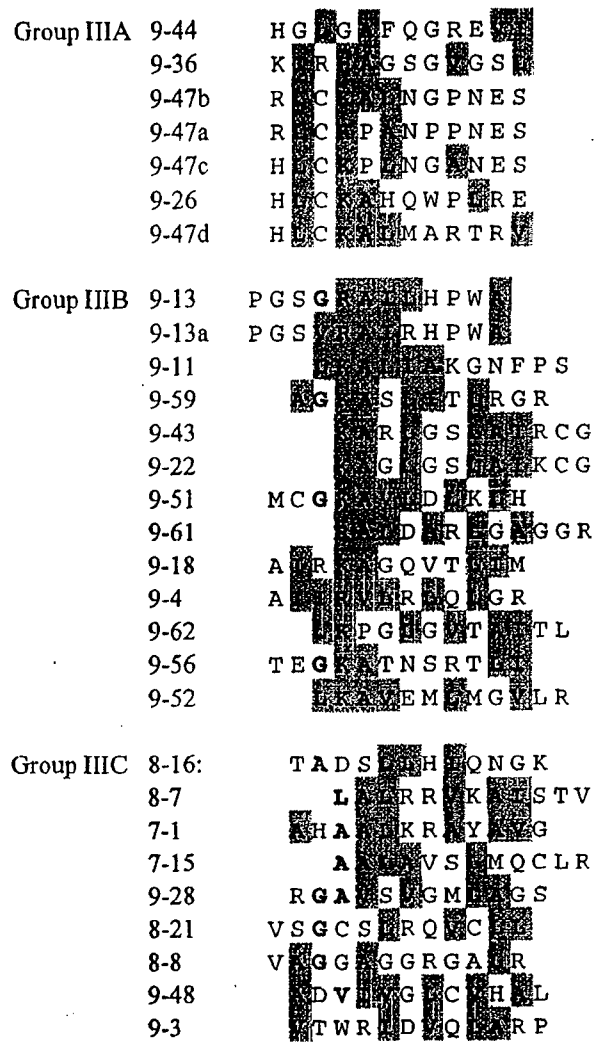
8-2	Y L G E Y L R S C W P G
8-9b	Y E G E Y T R S C W P G
8-9a	Y E G S T L R S C W P G
8-9	Y E G S T L P S C W P G

PROSITE search engine. Nearly 1500 hits were obtained, and among the hits were a number of enzymes/proteins related to the replication of human viruses (not shown).

A New PCNA-Binding Motif Found from the Isolates of the FliTrx Random Peptide Library. The third group of peptides that were aligned is shown in Table 2, and is subdivided into Groups IIIA, IIIB, and IIIC. This group consists of 29 members, about one-third of the total sequences that were isolated. Group IIIB contains 13 members, and is the largest of the 3 subgroups. Groups IIIA and IIIB are clearly related, while Group IIIC is more closely related to Group IIIB. The distinguishing feature of Groups IIIA and IIIB is the presence of a highly conserved KA pair, followed by two to four aliphatic hydrophobic residues that are mainly leucines. Group IIIA is distinguished from Groups IIIB and IIIC by the presence of a conserved pair of residues that consists of a basic residue often followed by leucine that is N-terminal to the KA pair that is absent in Groups IIIB and IIIC. Group IIIC is lacking the conserved basic residue of the KA pair that is present in Group IIIA and Group IIIB. The overall motif was termed the KA-box.

Attempts were made to search for related sequences using query patterns for the KA-box motifs in Groups IIIA and IIIB in the SWISSPROT database using the PROSITE search engine. However, because of the shortness of the sequence and a lack of very strong conservation, a large number of "hits" were obtained. For example, using a query pattern based on Group IIIA, over 600 hits were obtained using the PROSITE program. Using a shorter query pattern, based on Group IIIB, nearly 3000 hits were obtained. It is noted parenthetically that use of the PIP-box motif provides even larger numbers of hits. Included among the hits were a number of proteins that are involved with DNA replication, DNA repair, and cell cycle control. Among these were sequences present in the N-terminus of the catalytic subunit

Table 2: The Major Group of PCNA-Binding Peptides Isolated by Peptide Library Screening[a]



[a] Residues that are conserved over more than one group are shown in boldface and are shaded. Where a second residue in the same column is conserved, it is shown in boldface alone.

of pol δ. The region of similarity of the pol δ sequences falls in what is termed the N4 region by Yang et al. (*38*), and is conserved among pol δ enzymes from eukaryotes as well as in several viral polymerases including those of HSV, EBV, and CMV. An alignment of the N4 regions of pol δ of different species with members of the Group IIIA and IIIB peptides is shown in Table 3.

The Peptide, N4, Containing the New PCNA-Binding Motif Interacts with PCNA. In previous studies, we had examined the binding of PCNA to peptides conforming to conserved regions in the N-terminus of human pol δ, and have found that the N2 region, but not the N4 region, bound to PCNA by a dot blot procedure using a PCNA overlay method followed by detection with a monoclonal antibody against PCNA and a chemical staining procedure (*39*). For this reason, the issue of whether a peptide containing the N4 region would bind to PCNA was reinvestigated with the use of more sensitive detection methods. Dot blot experiments were performed in which samples of peptides were placed on nitrocellulose membranes and overlayed with biotinylated PCNA. The blot was visualized using a chemiluminescence method (see Experimental Procedures). A peptide corresponding to the N4 region of human pol δ p125 (276-RLKEKATQCQLEADVLWSDV-295) was tested together with peptides derived from other portions of the pol δ sequence (Figure 2). The peptides tested included three from the C-terminus (C1, C2, C3), and peptides based on the conserved N2 regions of pol δ. These were the N2 peptide (129-GVTDEGFSVCCHIHGFAPYFY-149), N2−6 (129-GVTDEGFSVAAHIHGFAPYFY-149), in which cysteines 138 and 139 were mutated to alanines and which has previously been shown to bind PCNA (*37, 39*), the two half-peptides of the N2 sequence, N2a (129-GVTDEGFSVC-138) and N2b (139-CHIHGFAPYFY-149), and N2AAA (129-GVTDEGFSVCCHIHGFAPAAA-149). Also included in these experiments were PCNA, purified calf thymus pol δ, and bovine serum albumin (BSA). The results showed that only peptides N4, N2, N2−6, and N2b gave positive signals (Figure 2). In the same experiment, positive signals were obtained with PCNA itself and with the pol δ enzyme but not with BSA. The difference between this experiment and our previous studies (*39*) is the use of a more sensitive chemiluminescence assay for binding, and suggests that the N4 peptide, while it does bind to PCNA, may not bind as strongly as the N2 peptide. It was also found that addition of the N4 peptide could successfully block the binding of biotinylated PCNA to the p125 catalytic subunit of pol δ in overlay experiments (data not shown).

A second experimental approach to confirm if these peptides could bind to PCNA was to test for their ability to inhibit the PCNA stimulation of pol δ activity. The results are shown in Figure 3. The N2 peptide, as a positive control, inhibits the PCNA stimulation of pol δ as we have previously shown (*39*). The N4 peptide also inhibited the PCNA stimulation of pol δ, as did the half-peptide N2b, although both of these were clearly less potent than the full-length N2 peptide. None of the other peptides tested (C1, C3, N2a, N2AAA) inhibited pol δ activity. The potency of inhibition of pol δ by N4 was roughly 5-fold less effective than the N2 peptide. These results demonstrate the ability of the N4 peptide to inhibit the PCNA stimulation of pol δ, consistent with its having the ability to bind to pol δ.

The demonstration that the C-terminal half, but not the N-terminal half of the N2 peptide, showed positive interactions with PCNA by inhibition assays provides additional evidence that the N2 peptide contains a variant of the PIP-box (*37, 39*). This is also consistent with the demonstration that the N2 peptide, but not the N2AAA peptide in which the aromatic resides of the PIP-box (*25, 26*) are mutated to alanine, is able to inhibit the PCNA stimulation of pol δ. Experiments in which other mutations of N2 (Figure 4) were tested showed that all these were able to inhibit pol δ activity, consistent with our previous data showing that these same peptides were able to bind biotinylated PCNA in overlay experiments (*37*).

A more direct test of the ability of the N4 peptide to bind PCNA was performed. The N4 and N2−6 peptides were immobilized on CH-Sepharose (see Experimental Procedures) and tested for their abilities to bind PCNA. Recombinant human PCNA was incubated with the peptide−Sepharose beads to which either N4 or N2−6 had been

Table 3: Alignment of the N4 Regions of the 125 kDa Subunit of Pol δ with the Group III Peptides[a]

N4 regions of pol δ:					
soy-bean		237	KTAKSLSYCQLEFDCL	252	AF020193
S. pombe		260	RYQNRVSNCQLEAWIN	275	L07734
EBV		259	RLQHRDSYAELEYDCE	274	V01555
hamster		272	RTEKKATQCQLEVDVL	287	AJ222691
rat		272	RAEKKATLCQLEVDVL	287	O54747
bovine		275	RPEGKATLCQLEADVL	290	M80395
human		276	RLKEKATQCQLEADVL	291	M01735
Peptides: IIIA	9-47b		RLC-KALNGPNES		
	9-47a		RLC-KPANPPNES		
	9-26		HLC-KAHQWPLRE		
	9-47d		HLC-KALMARTRV		
Peptides: IIIB	9-59		AGKASLITLRGR		
	9-18		AIRKAGQVTLIM		
	9-43		KARLGSLAIRCG		
	9-61		RALDARLGAGGR		
	9-52		LKAVEMLMGVLR		
	9-56		TEGKATNSRTLI		
	9-4		AIIRVLRLQLGR		

[a] Sequences from the N4 regions of pol δ are aligned with representative peptides from Groups IIIA and IIIB. Conserved residues are shown in boldface and are shaded. Accession numbers are given on the right.

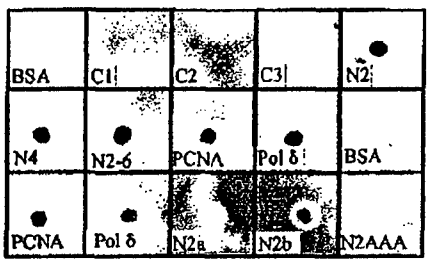


FIGURE 2: Dot blot analysis of the binding of PCNA to synthetic peptides. The synthetic peptides or proteins were dot blotted onto nitrocellulose and tested for PCNA binding by the use of biotinylated PCNA (Experimental Procedures). The blots were visualized using a chemiluminescence method. The synthetic peptides used were as follows: C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C2 (1069-VICTSRDCPIFYMRKKVRKDLED-1090), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N2−6 (mutant of N2, 129-GVTDEGFSVAAHIHGFAPYFY-149, CC → AA), N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAPAAA-149, YFY → AAA), and N4 (276-RLKEKATQCQLEADVLWSDV-295). BSA, purified calf thymus pol δ, and PCNA were also tested.

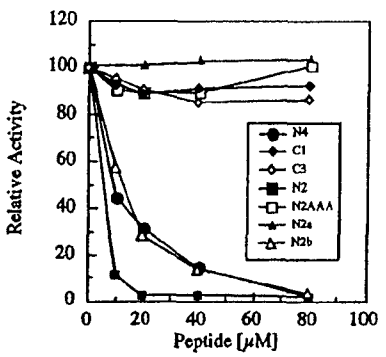


FIGURE 3: Inhibition of pol δ activity by the synthetic peptides N4 and N2. Human pol δ activity was assayed as described under Experimental Procedures in the presence of PCNA and varying concentrations (μM) of the peptides. Results are shown as relative activities. Pol δ synthetic peptides used in the assay include: C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N4 (276-RLKEKATQCQLEADVLWSDV-295), and N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAPAAA-149, YFY → AAA).

attached; the beads were washed, extracted with SDS buffer, and then Western blotted with antibody against PCNA (see Experimental Procedures). The results show that both the N4-Sepharose and the N2−6-Sepharose beads were capable of binding PCNA as shown by SDS−PAGE and silver staining (Figure 5). Similar tests of bovine serum albumin coupled to Sepharose were negative.

Both experiments, dot blot and adsorption of PCNA on N4- or N2−6-Sepharose, show that peptides N4 and N2−6 are able to bind PCNA. Zhang et al. (*37*) recently found that the N2 region of DNA pol δ could interact with PCNA. The peptide N2−6 is similar to its parent peptide, N2, in the ability of PCNA binding. The ability of N2−6 to bind to PCNA further verifies that the C-terminal portion of the conserved region in the N-terminus of DNA polymerase δ is involved in PCNA binding.

DISCUSSION

The results of this study show not only that sequences corresponding to the known PIP-box can be selected using a random peptide display library, but also that there is a novel family of peptides that bind to PCNA. This novel group comprised approximately one-third of the sequences isolated. While the alignment of these peptides did not provide a singularly tight consensus, the results reveal a novel peptide

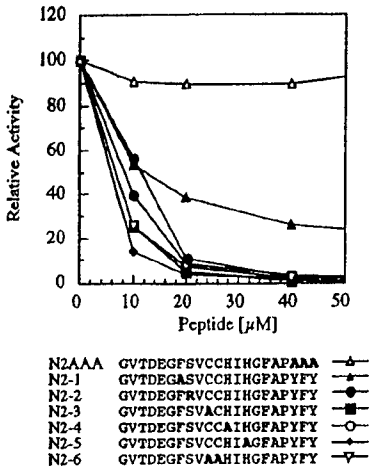


FIGURE 4: Inhibition of pol δ activity by the mutant peptides of N2. Human pol δ activity was assayed as described under Experimental Procedures in the presence of PCNA and varying concentrations (μM) of the mutant peptides of N2. Results are shown as relative activities. Pol δ synthetic peptides used in the assay are shown below the figure (mutated amino acids are marked in boldface letters).

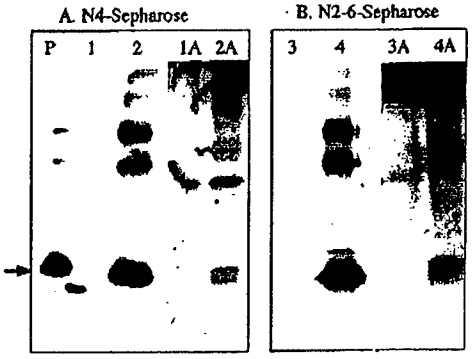


FIGURE 5: Binding of PCNA to immobilized N4 and N2−6 peptides. N4 and N2−6 peptides were covalently coupled to CH-Sepharose. The Sepharose beads (40 μL) were then used to bind purified recombinant human PCNA (40 μL, 0.1 μg/μL). After stringent washing to remove nonspecifically bound material, the beads were extracted with SDS-buffer and subjected to SDS−PAGE and Western blotting using an antibody to PCNA (see Experimental Procedures). Panel A: binding of PCNA to N4-Sepharose. Lane P, purified PCNA; lane 1, binding of PCNA to BSA−Sepharose; lane 2, binding of PCNA to N4-Sepharose; lanes 1A and 2B, silver stain of eluates corresponding to lanes 1 and 2. Panel B: binding to N2−6-Sepharose. Lane 3, binding of PCNA to BSA−Sepharose; lane 4, binding of PCNA to N2−6-Sepharose; lanes 3A and 4B, silver stain of eluates corresponding to lanes 3 and 4. The arrow marks the position of PCNA.

motif (KA-box) that is able to bind PCNA. This was confirmed by a positive overlay of the flagellin−thioredoxin fusion proteins with biotinylated PCNA. As a result of database searches, it was noted that the N4 region of pol δ contains a sequence that conforms to the KA-box. Experimental tests by the use of synthetic peptides in overlay and affinity chromatography experiments showed that the N4 peptide could bind to PCNA. This provides strong evidence that the N4 region of pol δ participates in PCNA binding, and also the first evidence for a functional KA-box in a PCNA-binding protein.

The identification of a KA-box in pol δ is of interest, since we have demonstrated by a number of experimental approaches that the p125 subunit of pol δ interacts with PCNA through a region identified as the conserved N2 region (*37, 39*) which contains a variant of the PIP-box. Thus, the implications of this study are that pol δ interacts with PCNA through the possession of at least two PCNA-binding motifs. A multisite interaction would favor formation of the pol δ−PCNA complex, since it would strengthen the association of the two proteins. Moreover, there is the additional possibility that these interactions could involve different PCNA molecules, since PCNA is a homotrimeric protein. This type of interaction could provide additional strength to the stability of a trimeric PCNA−pol δ complex. Indeed, there is some experimental evidence based on cross-linking experiments which indicates that p125 preferentially forms a complex with trimeric PCNA (*37*).

As already noted under Results, both the PIP-box and the KA-box motifs involve relatively short peptide sequences which do not have very strong signatures, so that database searching provides a large number of hits. This emphasizes the need for experimental verification of the functional ability of any proposed sequence in a given protein to participate in PCNA binding. The identification of a second motif that may be involved in PCNA binding is of particular significance, as it may be of utility in identifying PCNA-binding domains in candidate proteins that bind to PCNA. Furthermore, not all known PCNA-binding proteins contain the PIP-box sequence, and the existence of a second motif may provide an explanation.

The identification of PCNA-binding motifs is of significance since it would provide insights to the structural basis for the versatility of PCNA, given the current information that it is the nexus for multiple protein−protein interactions that underlie its participation in DNA replication, repair, and cell cycle control processes. A role for PCNA in cell cycle control has been indicated by the finding that cyclin/cdks formed quaternary complexes with PCNA and p21 (*19, 40, 41*). The interaction of PCNA with different cyclin/cdks during the cell cycle suggested that the regulated distribution of PCNA could be an important link to cell cycle control of DNA. p21 has been intensively studied since it was discovered to be an inhibitor of the of cyclin/cdks (*42*) and to be transcriptionally regulated by p53 (*43*). p21 inhibits pol δ in vitro and provides an attractive potential molecular mechanism whereby p53 induction could arrest DNA synthesis (*23, 44*) by competing for binding to PCNA (*45, 46*).

Recently, a number of additional proteins that bind to PCNA have been identified (*7−21, 24, 47−52*). These findings have major implications for understanding the role of PCNA. These PCNA-binding proteins fall into three major groups: DNA replication proteins—the catalytic and third subunits of pol δ (*10, 11, 37, 47−49*), RFC (*7−9*), pol ε (*50*), FEN1 (*12, 13*), and DNA ligase I (*14*); DNA repair proteins—XPG (*15*), uracil−DNA glycosylase (UNG2, *16*), mismatch repair proteins MSH2, MLH1, and PMS2 (*51*); cell cycle regulatory proteins—p21 (*22, 23*), p57 (*24*), and the cyclins (*19*). The use of PCNA affinity chromatography has also identified an association of a number of replication and repair proteins with PCNA (*47, 52*). A number of these have been shown to possess functional PIP-boxes, and sequence alignments have provided additional candidates for PCNA binding (*25, 26, 47−49*).

Table 4: Selected Proteins Containing Regions of Similarity to the N4 Regions of Pol δ and the Group III PCNA-Binding Peptides

Pol δ (N4 region)	276	RLKEKATQCQLEADVL	291	M01735
Pol ε	1533	KTICRAIQRFLLAYKE	1548	Q07864
RFC-140	682	KSSLKAIVAESLNNTS	697	P35251
RFC 38	221	RNLRKALLMCEACRVQ	236	P40938
RFC 37	244	GDLRKAITFLQSATRL	259	P35249
XPC	712	RA-RKARLAEPQLREE	726	Q01831
XPC (Mouse)	676	RA-RKARHLGAQLHDH	690	P51612
XPD	224	ELARKAVVVFDEAHNI	239	P18074
	612	HHYGRAVLMFGVPYVY	627	P18074
	630	SRILKARLEYLRDQFQ	645	P18074
XPG	116	RQAIKTAFRSKRDEAL	131	P28715
XPG (xenopus)	116	RQAIKAALSGNKQSNE	131	P14629
UNG2	74	IQRNKAAALLRLAARN	89	X15653
UNG2 (mouse)	56	IQRNKAAALLRLAARN	71	P97931
MCM3	311	DYVKKAILCLLLGGVE	326	P25205
MCM3 (S. cere)	374	DHIKKALLLMLMGGVE	390	P24279
MCM5	706	HAIHKVLQLMLRRGEI	721	P33992
MCM5 (S. cere)	746	LALDKALYALEKHETI	766	P29496
MCM7	348	EDVKKALLLLLVGGVD	363	P33993
MCM7 (xenopus)	347	EDVKKALLLLLVGGVD	362	Q91876
MSH6	724	AIFTKAYQRMVLDAVT	739	P52701
MSH6 (mouse)	721	AVFTKASQRMVLDAVT	736	P54276
Cyclin D3	92	VPTRKAQLQLLGAVCM	107	P30281
Cyclin D3 (mouse)	92	VPTRKAQLQLLGTVCL	107	P30282
BRCA 1	516	DFIKKADLA-VQKTPE	530	P38398
BRCA 1 (mouse)	509	DFIKKADSAGVQRTPD	524	P48754
BRCA 2	2725	WYAVKAQLDPPLLAVL	2740	P51587
BRCA 2 (mouse)	2646	WYAVKAQLDPPLLAVL	2661	P97929

[a] Conserved residues are shown in boldface and are shaded. Accession numbers are shown on the right, and proteins are those of human unless otherwise noted.

As already noted, database searches using the limited sequences of the PIP-box and the KA-box need to be treated with caution. Nevertheless, an intriguing number of proteins that contain the KA-box could be identified. Some of these are shown in Table 4. This list includes members of the MCM (mini chromosome maintenance protein) family, pol ε, three XP proteins (XPC, XPG, XPD), mismatch repair protein MSH6, cyclin D3, and BRCA1 and BRCA2. While the possibility that the KA-boxes in these proteins could serve as interaction sites for PCNA binding is purely speculative, evidence that these in fact interact with PCNA and the potential functional significance of such interactions need to be considered.

The nuclear uracil–DNA glycosylase (UNG2) that is involved in base excision repair has been shown to bind both PCNA and RPA (*16*). UNG2 contains a PIP-box at the N-terminus, and two binding sites for RPA (*16*). One of the RPA-binding sites is located between residues 7 and 18, and overlaps the PCNA-binding site. The other site is located between residues 73 and 90 and has the sequence RIQRNKAAALLRLAARNV. Curiously, this site consists of a KA-box (Table 4). Thus, UNG2 contains two sites for RPA, the first of which overlaps the PIP-box, and the second of which conforms to the PCNA-binding KA motif that is described here. Tests by Otterlei et al. (*16*) of the ability of N-terminally truncated UNG2 mutants to bind to PCNA by an ELISA method revealed that the PIP-box was the major determinant for PCNA binding by UNG2; nevertheless, a weak ELISA reaction was observed with a truncated UNG2 mutant in which the PIP-box had been deleted but which still contained the KA-box, leaving open the possibility that this might be due to PCNA interaction with the KA-box.

In summary, we have provided the first evidence for novel peptide motifs for PCNA binding. These findings point to potential relationships that may be important to explore, and also provide a starting point for site-directed mutational studies that could provide evidence for the identities of the PCNA-binding regions. Clearly, much further work needs to be done to establish the function of these motifs in PCNA binding.

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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